

Acta Haematologica

International Journal of Haematology
Journal International d'Hématologie
Internationale Zeitschrift für Hämatologie

Official Organ of the European Division of the International Society of Haematology

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Clinical Experience with Anticoagulant Therapy in the Management of Disseminated Intravascular Coagulation in Children

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Abstract. Within the last 10 years we have treated 31 patients: 3 with purpura fulminans, 17 with meningococcal sepsis and 11 with hemolytic-uremic syndrome who showed evidence of or were suggestive for disseminated intravascular coagulation (DIC). We have administered anticoagulant therapy to all of them. In purpura fulminans the presence of DIC and therapeutic effect venous heparin were undoubted. In meningococcal sepsis *in vitro* study suggests evidence of DIC to a certain extent but the benefit of heparin at first sight appears to be doubtful. By surveying our overall mortality in meningococcal infections, however, early heparinization of patients with severe fulminant meningococcal syndrome the *in vitro* coagulation studies are inconclusive and only indirect evidence of DIC can be demonstrated. The value of heparin and perhaps the addition of activators of fibrinolysis in this disorder is disputable.

It has been shown in animal experiments as well as in human pathology that disseminated intravascular coagulation (DIC) plays a significant role as a pathogenetic factor in several diseases, in particular in conditions in which the Sanarelli-Shwartzman phenomenon is an essential factor [7, 16, 23, 26, 30, 35, 36, 40, 45]. Regarding the clinical diagnosis of such disorders, however, more speculations than solid data have been published. The majority of the authors report single cases. Many of them consider autoimmune mechanisms as an important basic factor. More than 10 years ago we were impressed by some cases which showed typical features of DIC, but in one of them autoimmunity was extremely unlikely since the patient had a severe combined immunodeficiency (Swiss type of SCID). In an attempt to clarify this

Key Words
Anticoagulant therapy
Hemolytic-uremic syndrome
Heparin therapy
Intravascular coagulation
Meningococcal sepsis
Purpura fulminans
Sanarelli-Shwartzman phenomenon
Waterhouse-Friderichsen syndrome

controversy we, therefore, started a systematic study of unselected cases in 1960 with the following purposes (a) diagnostic to prove or to disprove DIC in living human beings, and (b) therapeutic to apply anticoagulation regularly and to standardize the procedure as rigidly as possible in order to evaluate the results in treated cases

Working hypothesis We assumed that in the following conditions DIC played a significant role purpura fulminans, meningococemia especially the Waterhouse Friderichsen syndrome, and the hemolytic uremic syndrome When one of these diagnoses was suspected, in addition to the usual antibiotic and supportive therapy a detailed program was immediately activated This comprises hematological and coagulation studies, and subsequently the institution of effective anticoagulation Preliminary reports on our observations were published in 1961 [41] and 1964 [24, 43] The present paper gives an account on our experience during this 10-year period of systematic study

Materials and Methods

The following patients were included in the study 3 with purpura fulminans 17 with meningococemia and 11 with hemolytic uremic syndrome

Diagnostic procedures In addition to the normal routine laboratory tests coagulation studies were carried out according to the methods described by DUCKERT [14] Fibrin split products were determined in the last 2 years Platelets were counted by a direct method with phase contrast

Therapeutic procedures Anticoagulation with heparin in a dose of $10\,000\text{ IU/m}^2/\text{d}$ e given 4-6 hourly intravenously was administered as soon as possible [24] As this measure was unsatisfactory in many cases in 1966 we increased the dose to $15\,000\text{ IU/m}^2/\text{die}$, infused continuously by means of an automatic adjustable micropump (Perfusor, manufactured by B Braun West Germany) Anticoagulation was considered to be satisfactory when the thrombin time was more than 60 sec with a normal control of 13-18 sec, and when the clotting time with a 5 time more concentrated thrombin solution did not exceed 15 sec In patients who failed to meet these criteria the heparin dose was accordingly increased or decreased in steps of at least 10% of the total dose Heparin was replaced by oral anticoagulants when long term anticoagulation was indicated No attempt was made to give platelets Fibrinogen deficiency if less than $60\text{ mg}\%$ was corrected by infusion of purified fibrinogen in a dose of 1 g/m^2 intravenously stat to be repeated if necessary until a level of approximately $200\text{ mg}\%$ had been reached We never tried to influence the patients natural fibrinolysis by using fibrinolysin or epsilon amino-caproic acid (EACA) Corticosteroids were purposely excluded in all patients

Results

Purpura fulminans The coagulation studies are presented in table I Case 1 was only partially studied by KOLLER *et al* [29] since at the time

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Table I Coagulation studies in 3 cases of purpura fulminans treated with heparin

Case	Age	Sex	Platelets $\times 10^9$	Fibrinogen mg %	Quick %	Factors, %				Results
						II	V	VIII	X	
1	5 years (29)	M	94	250	0	-	0	-	-	survived
2	15 months	M	48	500	42	80	43	41	62	survived
3	10 days (43)	M	93	70	34	21	45	41	44	died of staph. super infection

of its observation the techniques were not yet adequately developed. The same authors described a 2nd similar case with 43,000 platelets, 470 mg % fibrinogen and absence of factor V who received no anticoagulant therapy and succumbed within 1 week. Case 2, a 15-month-old boy was admitted with the typical picture of purpura fulminans (fig 1) and unequivocal coagulation defects. Case 3 presents the very rare occurrence of purpura fulminans in a newborn infant with 5 successive episodes. It was investigated thoroughly and published in 1964 [43].

The course of case 1 and 2 was favourable. They were treated with exchange transfusions using heparinized blood and in case 2 with an additional short term anticoagulation with heparin.

Case 3 unfortunately died of a staphylococcal superinfection. The histology of several biopsy specimens and at autopsy confirmed the presence of intravascular thrombi in different stages of organization of 17 cases (table II). They showed variable degree of changes. 10 cases had thrombocytopenia, 2 had normal and another 2 presumably normal platelet counts (estimated from the blood smears). In the last 3 no blood picture or thrombocyte counts are available. Fibrinogen levels were below 200 mg % in 6 cases. Other coagulation factors showed different anomalies in every case except the 2 who were not studied. In 5 out of the 7 last cases circulating fibrin split products could be demonstrated.

Therapeutic results seem to be unsatisfactory since 13 out of the 17 treated cases died. For comparison a survey of 41 patients with meningo-coecemia, treated during the same period, is given in table III. Out of 41



Fig 1 Purpura fulminans in a 15-month-old boy, showing typical skin lesions (case 2 table I)

living cases are described briefly, since their course is of considerable interest

Meningococcal sepsis and meningitis (Case No 6, table II) L U (J No 8598/63) 2 1/4-year-old boy On 19 5 63 he developed a sudden fever and vomited 20 h later he was admitted in severe shock and with wide spread petechiae, ecchymoses and necrotic skin lesions of various sizes Blood pressure 65/45 mm Hg There was no meningism, but the CSF was turbid with 83 cells, normal protein and slightly reduced glucose Throat swab, blood and CSF culture grew *N meningitidis* Coagulation factors were severely diminished, but fibrinogen level and platelet count were within normal ranges Treatment

Table III Meningococcal infections treated with or without heparin from 1960 to 1969

	Survived	Suc- cumbed	Total
<i>Patients treated with heparin</i>			
Meningitis	—	—	—
Sepsis and meningitis (purpura and shock present)	2	8	10
Sepsis without meningitis (WFS)	2	5	7
<i>Patients treated without heparin</i>			
Meningitis	71	1	72
Sepsis and meningitis (purpura but without shock)	16	1	17
Sepsis without meningitis (WFS)	0	6	6
			Total 112

with intravenous penicillin was begun along with heparinization for the first 36 h. Although the patient remained very ill for the first 3 days, his skin lesions subsided 48 h after admission and his blood pressure became normal within 24 h of treatment. On the 7th hospital day he started to show gradual improvement and he was discharged in good health after 29 days of hospitalization. He was seen again in the out patient department 5 years later and was found to be in good health both physically and mentally. Routine investigations were normal and X rays of his abdomen revealed no evidence of calcification in the region of the adrenals.

Meningococcal sepsis and meningitis (Case No 14, table II) G A (J No 0661/69), 15 month-old girl. On admission, 20 h after sudden onset of fever and vomiting, the child was in shock and presented with extensive purpura of the skin. Clinical and laboratory evidence of meningococcal sepsis and meningitis were present. In addition to the abnormal clotting factors as shown in table II, unequivocal diminution of factors VIII (27%) and IX (19%) were present. The immunoglobulins G, A and M were all reduced to about 50% of the normal values for her age. Immediate treatment consisted of penicillin i.v. and heparin. For the first 3 days the girl remained very ill, but on the 4th hospital day thrombocytes rose from 59,000 to 125,000 per mm³ and fibrinogen from 300 to 560 mg%. Heparin was discontinued after 4 days. The child made an uneventful recovery and was discharged home in good health and with no residual defects after 35 days in hospital. Her adrenal functions were normal 2 days after admission (normal plasma 11-OH-corticosteroid level) and shortly before discharge (ACTH test within normal limits).

Fulminant meningococcemia with Waterhouse-Friderichsen syndrome (Case No 7, table II) L II (J No 7939/63) 6-year-old female. Complained of shivering and vomited several times 12 h and became unconscious 3 h prior to admission. On examination there were generalized petechiae and ecchymoses. Blood pressure 70/30 mm Hg. Meningeal signs were absent. CSF contained 9 cells/mm³ and normal protein and sugar. *N. meningitidis* was cultured from CSF, throat swab, and blood. Coagulation studies were

compatible with DIC (table II). Treatment to combat shock was immediately instituted and antibiotics and heparin were given simultaneously. For the first 2 days the girl remained very ill, but became slightly responsive. On the 4th day she began to make gradual recovery, and after 5 weeks of hospitalization she was discharged home in good health. Heparin treatment was discontinued after 45 h. 5 years later (1968) the girl was seen again in the out-patient department. She has developed normally, done well at school and presented no anomalies of character. Routine investigations were within normal limits, and X rays showed no calcifications in the region of her adrenals.

Fulminant meningococemia with Waterhouse-Friderichsen syndrome (Case No 11, table II). S.R. (J No 590 69) 9 month-old male was admitted with 8 h history of fever and purpura. Clinical examination revealed generalized petechiae and ecchymoses. Blood pressure 80/0 mm Hg. There were no signs of meningitis. The CSF was normal. Meningitis was cultured in the CSF and blood. Results of coagulation study on admission are shown in table II. As in the other patients conventional treatment and anti-coagulant therapy was immediately instituted. The course at first was not favourable but after a fortnight the child started to show gradual improvement and could be discharged home in good health on the 34th hospital day. He was seen again in the patient department 3 months later and the only pathological finding was an abnormal EEG which indicates the presence of paroxysmal epileptogenic disturbance over the frontal regions. In plasma 11-OH-corticosteroid level on the 10th hospital day was normal.

Hemolytic uremic syndrome All 11 cases studied presented the typical features described first by GASSER *et al.* in 1955 [17]. The duration of illness immediately prior to admission varied from 24 h to 6 days, but this acute period was always preceded by gastrointestinal disorders. On admission, 4 children had hemoglobin levels below 4 g %, 2 had 4.0-5.0 and 5 had 6.0-6.0 g %. The characteristically shaped red cells (helmet forms or burr cells), hemoglobinuria, hemoglobinemia and or hematuria as well as reticulocytosis, were present in all cases. Clotting mechanisms were tested immediately after admission with the exception of cases 4 and 5 (see table IV). Thrombocyte counts were below 55 000/mm³ in all cases, except for cases 2 and 5 who had thrombocytes of 67 000 and 100 000 on admission but soon afterwards dropped to 4 400 and 24 000 per mm³ respectively. Coagulation studies are incomplete clear-cut defects are only found in 2 children (cases 2 and 8). The search for fibrin breakdown products in the last 4 cases was negative. All patients had high blood urea nitrogen ranging from 45 to 140 mg %. (normal 10-20 mg %). 6 had hypertension and 6 had aruna varying from 1 to 4 weeks of duration. 4 out of the 10 patients succumbed but only 1 died in the acute state. The other 3 died of renal and eventually cardiac failure one 2 and 3 months after the onset of the disease. Among the 5 survivors only 1 suffers from a secondary cerebral palsy and has persistent proteinuria. The remaining 3 children recovered

Table IV Coagulation studies in hemolytic uremic syndrome treated with heparin from 1960 to 1

Cases	Age	Sex	Plat- elets on admis- sion $\times 10^3$	Fibri- nogen mg %	Quick %	Factors, %				Fibrin split products	Resul- Survived
						II	V	VII	X		
1	8 months	F	40	-	-	-	-	-	-	-	+
2	10 months	M	100	170	56	80	90	80	90	-	
3	2 months	M	29	-	-	-	-	-	-	-	+
4	4 years	M	32	240 ¹	80 ¹	-	75 ¹	-	81 ¹	-	+
5	7 years	F	67	220 ¹	100 ¹	-	-	-	-	-	
6	4 months	M	54	270	72	-	100	-	-	negative	+
7	14 months	M	36	440	70	-	100	-	-	negative	
8	1 year	M	18	140	28	26	100	9	11	negative	
9	3 1/2 years	F	12	175	92	-	-	-	-	negative	+
0	9 months	F	31	-	77	-	-	-	-	-	+
1	2 1/4 years	F	17	200	57	-	-	-	-	-	+

¹ Coagulation studies performed several days after heparin therapy

completely with normal blood pressure and urine. Two of them who had a continuous follow-up study have normal renal function tests, 1 to 7 years after their illness. The course in 1 of the surviving children, a 4-month-old boy, is shown as an example in figure 2.

Discussion

Evidence of DIC. Morbid anatomists pointed out the occurrence of numerous thromboses in small vessels of patients who died of the disorders discussed here. They showed that the ensuing ischemia causes necroses in organs, especially in kidney, brain, liver, and heart [11, 16, 17, 23, 25, 26, 29, 30, 35, 36, 40, 45]. Systematic studies of similar conditions experimentally produced in animals demonstrated clear-cut diminution of coagulation factors in a regular, rapid sequence: the initial phase is characterized by a drop of platelets and factors II, V, VII, VIII, X. In the 2nd phase most of these factors may rise to normal levels, except the platelets, at this stage fibrinogen may drop to very low levels, leading to afibrino-

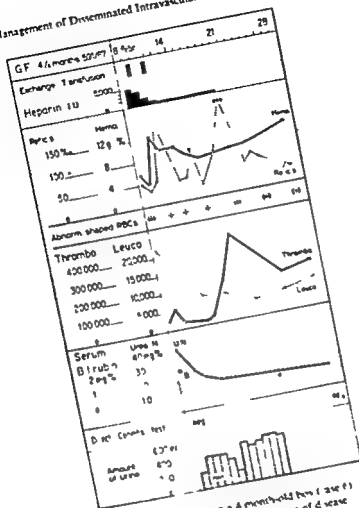


Fig 2 Hemolytic-uremic syndrome in a 4 month-old boy (case 1) clinical and laboratory findings, treatment with antithrombin and course of disease

genemia. The 3rd phase is characterized by activation of fibrinolysis with red resolution of thrombi and this may be demonstrated by the presence of fibrin split products in the circulation. This evolution shows that proper repair and repair of DIC are going on simultaneously. Therefore a steady state is never reached and the levels of single coagulation factors can vary rapidly.

Limited experience in human beings confirms essentially the same sequence of events [7, 26, 30, 35]. Apparently, the platelets are most sensitive and thrombocytopenia is therefore a reliable sign for the occurrence of DIC, even when the other clotting factors are already normalized [27]. The diagnostic value of clinical findings is more difficult to determine, but we feel that the easily recognized signs of vascular obstruction and ischemia present strong evidence for DIC, especially when skin and kidneys are involved.

Clinical occurrence of DIC in pediatrics A long list of disorders with concomitant DIC has been presented by several authors [1, 7, 16, 23, 25, 30, 36]. However, convincing evidence is lacking except for the 3 diseases which are the subject of this report. In all of them conventional therapy was either undisputed (e.g. antibiotics in meningococemia) or questionable (e.g. corticosteroids), but not satisfactory, since it did not take into account DIC. Endotoxins probably initiate the whole process, although the exact mechanism is not yet clear. In adults the damage inflicted by an endotoxin on the vascular intima can be shown by counting the number of endothelial cells circulating in the blood [9], but in pediatrics no such experience exists. Local endothelial lesions are probably the starting point for an activation of the clotting mechanism, which later on becomes self-perpetuating.

Purpura fulminans There is general agreement that small blood vessels especially of the skin and subcutis are occluded by thrombi. DIC has been documented by numerous *in vivo* coagulation studies in the literature [1, 3, 4, 8, 27, 31, 36] as well as by our 3 cases. Indirect evidence for the importance of DIC was derived from the benefit of anti-coagulation as applied essentially by the same authors. Additional cases in which simple blood transfusion has been claimed to influence the course of this disorder [8] may be mentioned in this connection, since the short period of involuntary administration of an anticoagulant with the transfused blood might be sufficient to break the vicious circle of continuing DIC.

On the other hand we must mention here exceptional single cases with convincing improvement after the administration of high doses of corticosteroids [6, 13, 44] or of dextran [37]. We are unable to offer an explanation for these cases.

Meningococcal sepsis The main difficulty in this condition results from its variegated clinical appearance which in turn probably is a consequence of the degree of imbalance between macro- and micro-organism. The cases of 'healthy' carriers of bacteria are well known, less is known about the causes of septic generalization – one possible factor being antibody de-

iciency [28], there is total ignorance concerning the factors leading to peracute septic generalization. In this latter condition, however, the simultaneous occurrence of shock and DIC is very important for the pathogenesis of the subsequent usually lethal course. Severe shock was observed in about $\frac{1}{3}$ of our cases (23 out of 112, table III). More than half of these patients namely 13, presented the most severe course with peracute overwhelming septicemia but almost no signs of defense reactions of the macro-organism: no pleocytosis in the CSF, nor leucocytosis in the blood despite of massive bacterial growth in cultures. This clinically important combination is defined as Waterhouse-Friderichsen syndrome by FANCONI and WALLGREN [15]. At necropsy the hemorrhagic-necrotic lesions of the adrenals are the prominent feature.

We want to stress the fact that only these very severely ill patients with virtually infaust prognosis were selected on clinical grounds for anticoagulant treatment in addition to the usual therapy. As demonstrated in table II all the 15 patients who could be investigated showed some evidence of DIC which admittedly is not in every case very striking. 10 out of 12 patients had definite thrombocytopenia, diminution of coagulation factors were found in every case, and 5 of the 7 last cases had circulating fibrin split products.

Success of treatment seems poor at first sight, since only 4 patients out of 17 survived. However in view of the peracute and rapid course of the disease, this seems an unequivocal improvement. As may be seen from table III there were 6 additional patients with WFS who died immediately after admission and before any treatment could be instituted. We are, therefore, convinced that the 4 surviving children were saved by the addition of anticoagulants to the conventional therapy. Careful documentation of such cases as given earlier in this paper is necessary to evaluate the extent of shock and defense reaction. In particular this is true for the cured patients with presumed WFS since up to the present time this diagnosis could only be made on the ground of the adrenal hemorrhages at post mortem examination.

A few other cases have been published. McGRATH *et al* [34] administered heparin to 5 of 6 patients with fulminant meningococemia, but only 1 early treated case survived. CORREIA *et al* [12] have reported more favourable results: among 4 patients with meningococemia who showed evidence of DIC and received heparin there were 3 survivors. Two other cases of meningococemia have been successfully treated by anticoagulation [2, 42], 1 of them fulfils all the criteria according to FANCONI and

WALLGREN [15], for the *in vivo* diagnosis of the Waterhouse-Friderichsen syndrome. It is, therefore, likely that in all these 10 very severely ill patients the rapid progression of the disease was interrupted by the administration of heparin.

There is still controversy as to the value of corticosteroid therapy. MARGARETTEN and MCADAMS [32] and MARGARETTEN *et al* [33] presented evidence in their extensive material that the number of fatalities amongst patients with meningococemia had considerably increased since the routine administration of cortisone. This might be explained by the well-established fact that the experimental Sanarelli-Shwartzman phenomenon is enhanced by corticosteroid treatment [20]. At the beginning of our study we, therefore, decided to avoid steroids.

The authors [1, 6, 21] who recommend the use of corticosteroids claim that they are necessary either for replacement of the destroyed adrenals or to combat shock. The first argument is borne out by the – rare but unequivocal – findings of normal 11-OH-corticosteroids in blood, the normal response to ACTH and the complete recovery in the cured patients. The 2nd goal – repair of shock – is undoubtedly of utmost importance, but it can better be reached by other means. Our own experience may not completely settle the question whether corticosteroids are useful, but it shows clearly that they are not indispensable.

Hemolytic-uremic syndrome: The diagnosis of hemolytic-uremic syndrome is primarily based on clinical signs and pathological findings in blood and urine. In patients who die within the 1st days, extensive thrombi in small vessels suggestive for DIC have been found, especially in kidney, brain, heart, liver, spleen, and adrenals [17]. Scepticism has been expressed on the ground of histological findings in patients who died several weeks or months after the acute episode of the disease, and instead the presence of glomerular angiopathy was claimed [22]. As mentioned before, at this late stage of the disease the primary lesions and damages of DIC are overshadowed by subsequent repair processes. Renal biopsy in the acute stage alone could help to clarify this controversy, but in practice it is not feasible.

In vivo diagnosis of DIC is difficult to confirm besides variegated signs of organic ischemia (kidney oliguria, brain convulsions and hemiplegia, skin hemorrhagic infarctions) the signs of microangiopathic hemolytic anemia [5, 10, 39] with numerous burr cells, etc. and of thrombocytopenic purpura are present.

The laboratory coagulation data, however, in our 11 cases do not unequivocally confirm the presence of DIC at the time of the study only.

thrombocytopenia is a constant finding but diminution of specific coagulation factors is irregular or even absent. All these findings seem to indicate that the patients were admitted in a late phase when a repair of most damages except thrombocytopenia was already taking place. If this is correct, however, the presence of fibrin split products should be expected and this was not the case in all the 4 patients studied in this respect. We have therefore, the impression that only indirect evidence of the *in vitro* studies is in favour of DIC.

Our results of treatment including heparin administration are again at first sight not very spectacular, since 4 of the 11 patients died. It compares unfavourably with GIANANTONIO *et al* [18] who collected 240 cases in a 10-year period with only 16% mortality or with PIRL and PIRIBS [35] and GILCHRIST *et al* [19] who had more favourable results with heparin (2/3 and 4 cases, respectively saved). By comparison of the lowest hemoglobin and thrombocyte counts it becomes evident that all our patients were severely affected which was only exceptioned in the South American series [18]. Groups of patients treated by different procedures should therefore not be compared without considering these differences in severity.

The 2nd factor time has already been mentioned. All the patients were admitted to hospital when the signs and symptoms of a severe disease were already fully established but again the extent of the damage varied greatly from one individual to the other.

Rationale of heparin therapy. The acute course of the above listed conditions precludes anticoagulant therapy before the development of ischemic lesions in different organs. It is only possible to prevent further activation of the clotting system and deposition of more fibrin within the lumina of blood vessels by short term anticoagulation from 2 to 7 days.

The addition of fibrinolytic agents to the anticoagulants seems to be logical but we have not attempted to carry out this measure in a state of flux which is already too complicated to be fully understood. In any event the use of fibrinolytic therapy will require specialized laboratory guidance.

Practical recommendations for therapy. According to the results of our 10 years of study it is undoubted that there is a place for heparin treatment in childhood illness with evidence of or suspicion for DIC and this has to be conducted in the first urgency. At present we recommend a dose of 1500 IU per m² body surface per day given continuously with an automatic pump. In purpura fulminans anticoagulation is definitely effective. Early heparinization of patients with fulminant meningococemia in severe shock is of value in modifying the course. In hemolytic uremic syndrome

administration of heparin is justified in order to prevent or modify cellular death following vascular and capillary thrombotic occlusions in various organs, particularly in the kidneys

However, further clinical experience and laboratory studies should be sought for a definite recommendation regarding the therapeutic effectiveness of anticoagulation and/or fibrinolytic agents in this at present much disputed disorder. It seems thus reasonable to plan and conduct a cooperative study with several participating medical centers in an attempt to solve this problem.

Acknowledgments. This work was supported by 'Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung'.

We would like to thank Drs W. W. ZUELZER and H. LEUMANN for advice and criticism and Dr A. FANCONI for permission to study 2 cases of meningococcemia under his care, Dr O. TRÜEB for following up the patient G. F. with HUS and carrying out the renal function tests on him, and Dr R. LANDOLT for allowing us to study 1 case of HUS (case No. 10) under his care.

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Autoradiographic Studies of RNA Metabolism in Human Leukaemic Blast Cells

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Abstract. In an autoradiographic investigation it was found that the inhibition of RNA synthesis in human leukaemic blast cells by actinomycin D (AMD) is dose and time dependent. Chase studies with AMD showed that most of the rapidly labelled RNA is unstable. The decay pattern is biphasic, being most rapid in the initial 30-40 min and much slower thereafter. Long lived templates for protein synthesis were not revealed by the present study.

Key Words:
Actinomycin D
Autoradiography
Cell culture
Leukaemic cells
RNA synthesis

RNA and protein synthesis are closely related to processes controlling proliferation in a variety of cell types [1] and may therefore be of importance in the study of cell proliferation in leukaemia. Some critical steps in the cell cycle involve the synthesis of short lived messenger RNA and are promptly inhibited by actinomycin D (AMD) [2, 3, 4], an antibiotic which specifically inhibits the transcription of DNA to RNA [5].

This work was designed to investigate (a) the dose response of the inhibitory effect of AMD on RNA synthesis in human leukaemic blast cells, (b) the rate of decline of RNA radioactivity following different exposure times to a labelled RNA precursor, (c) the rate of degradation of rapidly labelled RNA in cells from acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) and (d) the effect of AMD on protein synthesis in leukaemic blast cells.

Methods

Cells for culture were obtained from the marrow or blood of patients suffering from AML (19 cases) and ALL (16 cases). All samples had more than 85% blast cells. The culture medium contained FC-127 (Gibco) 10% and AB Rh+ve serum or anti-human plasma 5%.

Uridine 5 ^3H (^3H UR), 2 $\mu\text{Ci/ml}$, specific activity 5 Ci/mM was used as the labelled RNA precursor. A preliminary experiment using nuclease digestion, established that most of the label appeared in RNA and less than 3% in DNA. ^3H Leucine (^3H Leu), 10 $\mu\text{Ci/ml}$, specific activity 11.5 Ci/mM was used as the labelled amino acid. Following exposure to AMD (Merck, Sharpe and Dohme Ltd.) and/or radiochemicals (Radiochemicals Centre, Amersham) as described below, the cultures were sampled and processed for autoradiography using Ilford G5 emulsion. After a suitable exposure time the autoradiographs were developed and fixed. Grain counts were made on 50–100 cells.

Results

The inhibition of ^3H -UR uptake was studied using a range of AMD dosage. 7 cultures were prepared from each of 3 leukaemic cell samples. AMD was added at concentrations of 0, 0.01, 0.04, 1.0, 4.0 and 10.0 $\mu\text{g/ml}$. After 1 h incubation with AMD, ^3H -UR was added and incubation was continued for a further 30 min. The cultures were then processed for autoradiography. In the control cultures 85–98% of cells were labelled. Grain counts in the other cultures were expressed as percentage of that of the control cultures (fig. 1). ^3H -UR uptake was virtually completely suppressed at AMD concentrations of $> 4.0 \mu\text{g/ml}$.

The effect of AMD at concentrations of 0, 0.4, 4.0 and 10.0 $\mu\text{g/ml}$ was next compared in experiments in which cultures were first exposed to ^3H -UR for 30 min prior to the addition of AMD. Incubation was continued for 4 h after addition of AMD, without changing the culture medium. There were 5 control cultures (not treated with AMD), 3 cultures at 0.4

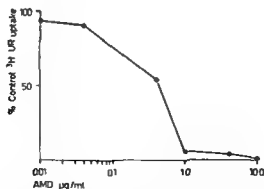


Fig. 1 Inhibition of RNA synthesis produced by different concentrations of AMD

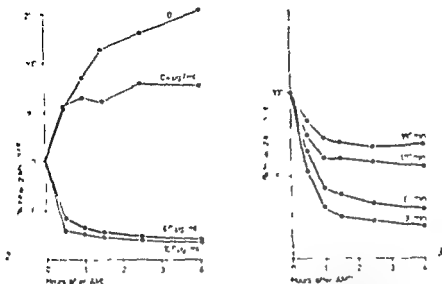


Fig. 2. Chase experiments showing changes in RNA radioactivity in cultures incubated with $2 \mu\text{Ci ml}^{-1}$ ^3H UR for 30 min prior to the addition of AMD at dosage shown.

Fig. 3. Comparison of 4 pre-incubation times with $2 \mu\text{Ci ml}^{-1}$ ^3H UR before addition of $4 \mu\text{g ml}^{-1}$ AMD.

$\mu\text{g ml}^{-1}$, 18 cultures at $40 \mu\text{g ml}^{-1}$ and 5 cultures at $100 \mu\text{g ml}^{-1}$ of AMD. The cultures were sampled at intervals and processed for autoradiography. Grain counts were expressed as the percentage of grain count in the initial sample, i.e., the sample taken just before addition of AMD (fig. 2).

Although in the first $\frac{1}{2}$ h, cultures treated with $0.4 \mu\text{g ml}^{-1}$ AMD did not differ significantly from the control cultures, after $\frac{1}{2}$ h the 2 graphs diverged considerably. Thus the effect of AMD at this relatively low dose was time dependent. Cultures treated with 40 and $100 \mu\text{g ml}^{-1}$ AMD showed a rapid decline in RNA radioactivity the first $\frac{1}{2}$ h, thereafter it declined more slowly. The decline was greater in the cultures treated with $10 \mu\text{g ml}^{-1}$ AMD but one such culture showed an almost complete loss of radioactivity with also morphological evidence of cell damage. This was possibly due to a general toxic effect of AMD. For this reason the concentration of $40 \mu\text{g ml}^{-1}$ was chosen for subsequent experiments.

The effect of different incubation times in the presence of ^3H UR prior to the addition of AMD was next studied. In one experiment, 4 cultures

were prepared from the same leukaemic cell samples. At 30, 60, 120 and 180 min after the start of incubation with ^3H -UR, 4.0 $\mu\text{g}/\text{ml}$ AMD was added. The cultures were then sampled at intervals during the next 4 h (fig 3). Comparison was also made between experiments in which the incubation time with ^3H -UR prior to adding AMD was 30 min (18 experiments) and 180 min (4 experiments). Results are shown in figure 4. The decline in RNA radioactivity revealed by these chase studies was dependent on the incubation time with ^3H -UR prior to adding AMD, the decline being greatest with the shortest incubation times.

The combination of short incubation time with ^3H -UR and a dosage of AMD which virtually completely inhibits RNA synthesis, provided the best conditions for observing the fate of rapidly-labelled RNA. Incubation time of 30 min and AMD dosage of 4.0 $\mu\text{g}/\text{ml}$ were chosen for further experiments. Preliminary studies have shown that addition of carrier uridine or the change of the culture medium, both done at the same time as the addition of AMD, did not significantly influence the result in studies of the rate of degradation of rapidly labelled RNA in leukaemic cells [personal data].

Comparison was made between studies on 9 patients with AML (fig 5) and 9 studies on 6 patients with ALL (fig 6). There was no statistically significant difference in the proportion of unstable, rapidly-labelled RNA between the 2 cytological types of acute leukaemia.

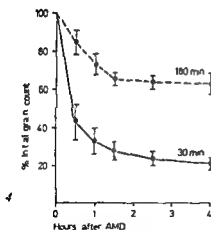


Fig 4 Comparison of pre incubation times of 180 min and 30 min (mean \pm SD)

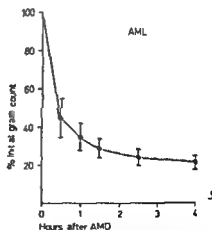


Fig 5 Chase experiments using AML cells with incubation time 30 min (2 $\mu\text{Ci}/\text{ml}$ ^3H UR) prior to addition of 4 $\mu\text{g}/\text{ml}$ AMD (mean \pm SD)

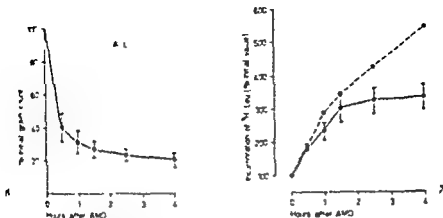


Fig. 6 Chase experiments using ALL cells with experimental conditions as for figure 5 (mean \pm SD)

Fig. 7 The effect of AMD on further incorporation of ^3H leucine in leukaemic blast cells (mean \pm SD): --- Control, — with AMD

The effect of AMD on protein synthesis was next studied. 6 cultures were incubated with ^3H -Leu for 1 h, 40 μg /ml AMD was then added and incubation was continued for a further 4 h. Three similarly incubated cultures, but without the addition of AMD, acted as controls. The graph for the incorporation of ^3H Leu in AMD treated cultures continued to rise for 1 h after the addition of the antibiotic, thereafter it flattened out markedly (fig. 7). In the control cultures, ^3H Leu incorporation continued throughout the period of observation.

Discussion

It is concluded that the majority of human leukaemic blast cells in short term *in vitro* culture synthesise RNA. This synthesis is DNA dependent as shown by the effect of AMD. The present work, therefore, does not support the thesis that these cells have an RNA-directed RNA synthesis, possibly of viral origin, which has been put forward [6, 7]. The inhibition of RNA synthesis by AMD as well as being dose dependent, is also time dependent. A relatively low dose of AMD (0.4 $\mu\text{g}/\text{ml}$) did not interfere with the intracellular RNA turnover during the first 1 h, but did so significantly during the next 3 h of observation. This suggests that part of the RNA synthesis is indirectly controlled by recently synthesised RNA which might be particu-

cularly sensitive to AMD. An example of this type of control is that part of the newly synthesized RNA may be required in a sequence of biochemical steps necessary for the flow of cells through the cell cycle. Arrest of cells, for example within G_1 by the small dose of AMD, could thus explain the reduction in RNA turnover.

This study shows that most of the rapidly-labelled RNA is unstable. The decay pattern is biphasic, being most rapid in the initial 30–60 min, and much slower thereafter. This conclusion contradicts that of STORTI and TORELLI [8] but is in agreement with most biochemical work [9, 10, 11]. Long-lived templates for protein synthesis were not revealed by the present study.

Acknowledgement I thank Prof F G J HAYHOE for advice and encouragement and Mr R J FLEMANS and Miss C SMITH for technical assistance. This work was supported by an Elmore Research Studentship.

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Zytologischer Immunglobulinnachweis an DNS-synthetisierenden Blutlymphozyten von Patienten mit Virusinfektionen

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Abstract. On 6 patients suffering from rubella or morbilli, we examined the portion of IgG-containing cells and the number of DNA-synthesizing lymphocytes repeatedly during the course of the disease using immunofluorescent and autoradiographic methods. In virus infections we found a marked increase of both cell populations by comparison with normals. During the course of the disease we observed a simultaneous increase and decrease of both IgG-containing and DNA-synthesizing lymphocytes. At the beginning of that reaction, DNA-synthesizing cells prevailed upon IgG-positive cells. In 2 cases, a small portion of IgG-containing cells was found to be in the stage of DNA-synthesis.

Key Words:
Autoradiography
DNA-synthesis
IgG in lymphocytes
Immunocytochemistry
Lymphocytes
Virus diseases

Während sowohl IgG-haltige als auch DNS-synthetisierende Lymphozyten beim Normalen nur jeweils in der Größenordnung um 10^6 beobachtet werden [1, 2] wurde eine starke Vermehrung beider Zellgruppen bei verschiedenen Viruserkrankungen beschrieben [1, 2, 3]. Es schien daher von Interesse, bei solchen Infektionen in zeitlichen Verläufen sowie durch kombinierte Darstellung zu untersuchen, in welchem Verhältnis beide Zellpopulationen zueinander stehen.

Material und Methodik

Es wurde die Lymphozytenzahl von 3 Patienten mit Rubella (Patient H.G., 19; Patient H.F., 24; Patient H.D., 19) und 3 Masernerkrankten (Patient R.M., 24; Patient P.F., 7; Patient A.B., 9) während 2 bzw. 3 wöchentlichen Abständen untersucht. Jeder von 6 Patienten wurde am 1., 2., 4. und 6. Tag sowie 2 Patienten am 9. Tag nach Erkrankungsbeginn jeweils am Morgen (9-11)

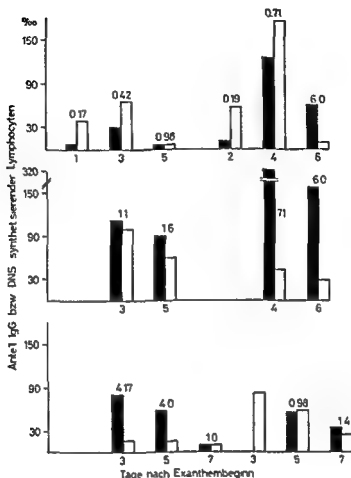


Abb 1 Zahl IgG haltiger (dunkle Säulen) und DNS synthetisierender Zellen (helle Säulen) im Krankheitsverlauf von 3 Rubeolenpatienten (links) und 3 Masernkranken (rechts) Der Quotient aus dem Anteil IgG positiver Zellen zur Zahl DNS synthetisierenden Zellen ist oberhalb der Säulen eingetragen

Methoden Heparinisiertes Venenblut wurde spontan sedimentiert und mit ^3H Thymidin ($1\mu\text{Ci/ml}$ Kultur spez Akt 5000 mCi/mm Markierungsdauer 60 min bei 37°C) inkubiert Autoradiographisch wurde der Anteil ^3H Thymidin einbauender Lymphocyten [Methode 4] sowie immunfluoreszenzoptisch die Zahl IgG haltiger Zellen [Methode 1] bestimmt Zu jeder Untersuchung wurden mindestens 2000 Lymphocyten ausgewertet Auf eine Inkubation mit Anti IgA und Anti IgM wurde verzichtet da wie früher von uns und anderen berichtet [1-3] die Anteile IgA bzw IgM positiver Zellen niedriger lagen

Das Ergebnis der immunzytologischen Untersuchung wurde in 2 Fällen (Patient II M Patient H G) photographisch festgehalten die Präparate anschliessend autoradiographiert und die Ergebnisse beider Methoden über die Photographien korreliert

Ergebnisse

Beim Normalen fanden wir im Mittel weniger als 10^6 IgG positiver [1] und $1,3^6$ an DNS-synthetisierender Lymphozyten [4] während diese Indizes bei den untersuchten Patienten mit Masern- bzw. Rubeoleninfektionen am Höhepunkt der Reaktionen das 12- bis 320fache bzw. das 17- bis 175fache der Normalen betrug.

In allen Verlaufbeobachtungen war die Zahl IgG haltiger Zellen dem Anteil von Lymphozyten in DNS-Synthese deutlich korreliert, jedoch überwogen die letzteren am Exanthebeginn (1 und 2 Exanthemtag). So betrug der Quotient aus der Zahl IgG positiver Zellen zum Anteil von Lymphozyten mit nachweisbarem ^3H -Thymidineinbau zu diesem Zeitpunkt weniger als 0,2 während bei allen späteren Untersuchungen Werte zwischen 0,4 und 7,1 beobachtet wurden (Abb. 1). Bei zwei Kranken, die bereits am Exanthebeginn zur Auswertung kamen, fanden wir die höchsten Anteile beider Zellgruppen am 3 bzw. 4 Tag nach Exanthemausbruch. Bei den übrigen Patienten, die zu einem späteren Zeitpunkt erstmals untersucht wurden, konnten keine Gipfel abgegrenzt werden, da die Reaktionen bereits im Abklingen begriffen waren. In zwei Fällen näherten sich die Anteile beider Zellpopulationen bereits am 5 bzw. 7 Tag nach Ausreten des Exanthems den Normalwerten, während in den anderen Fällen am 4., 6., 7. und 8 Tag noch deutlich erhöhte Indizes für beide Gruppen festgestellt wurden.

Sowohl bei den DNS-synthetisierenden Lymphozyten, wie auch bei jenen mit nachweisbarem IgG Gehalt handelte es sich um Zellen sehr verschiedener Größe. Die Mehrzahl der ^3H -Thymidin markierten Lymphozyten waren grosse basophile Mastenähnliche Zellen. Bei den IgG haltigen Zellen waren dagegen Plasmazellen sowie mittelgroße und kleine Lymphozyten am häufigsten. Immerhin fanden wir, dass ein kleiner Teil der IgG positiven Zellen an Größe, Kernform und Kern-Plasma-Relation den DNS-synthetisierenden vergleichbar waren.

In zwei Fällen wurde mittels einer Kombination immunzytologischer und autoradiographischer Methoden geprüft, ob im Rahmen einer Reaktion Lymphozyten mit nachweisbarem IgG Gehalt und gleichzeitigem ^3H -Thymidineinbau beobachtet werden können. Dabei fanden wir, dass sich 14% (Patient R.M.) bzw. 17% (Patient H.G.) der IgG positiven Zellen im Stadium der DNS-Synthese befanden. Bei welchen Lymphozyten, an denen beide untersuchte Eigenschaften nachgewiesen werden konnten, handelte es sich vorwiegend um größere Zellen.

Diskussion

Mit immunzytologischen Methoden können an fixierten Zellstrichen in etwa 10% der mononukleären Zellen Immunglobuline nachgewiesen werden [1]. Eine starke Vermehrung dieser Zellen bei Virusinfektionen wurde beschrieben [1, 3]. Autoradioimmunelektrophoretische Untersuchungen an Kulturüberständen von Zellen, die *in vitro* mit markierten Aminosäuren inkubiert wurden, waren den Ergebnissen der immunzytologischen Methode vergleichbar [3]. Soweit diese letztere Technik morphologische Aussagen zulässt, ist die Mehrzahl der IgG-haltigen Zellen den kleinen und mittelgrossen Lymphozyten sowie den Plasmazellen zuzordnen, während nur ein geringerer Teil grössere Zellformen umfasst [1, 3].

Mit autoradiographischen Methoden fanden sich an Normalen weniger als 20% DNS synthetisierender Lymphozyten [2, 4]. Bei verschiedenen Erkrankungen [4, 5], insbesondere bei Virusinfektionen [2, 5] wurde eine starke Zunahme dieser Zellen beobachtet. Bei der Mehrzahl von ihnen handelt es sich um grosse, blastenähnliche Zellen. Nur ein geringer Teil (etwa 1/3) ist kleiner und morphologisch den Plasmazellen und typischen Lymphozyten vergleichbar [5].

Da DNS synthetisierende wie IgG-haltige lymphatische Zellen bei Virusinfektionen vermehrt auftreten, untersuchten wir das Verhältnis dieser Populationen zueinander im zeitlichen Verlauf. Dabei zeigte sich, dass in dieser Hinsicht die Zahl IgG-haltiger Zellen den DNS synthetisierenden Lymphozyten deutlich korreliert war. Allerdings überwogen in den ersten Tagen nach Exanthemausbruch die ³H-Thymidin-inkorporierenden Lymphozyten über jene mit nachweisbarem IgG-Gehalt. Es lag daher nahe, in den ersteren Vorstufen IgG-positiver Zellen zu sehen. Dass solche Vorstufen nach Immunisierung vermehrt in den Lymphknoten auftreten, konnte im Tierexperiment gezeigt werden. So fanden BALFOUR *et al.* [6] nach Immunisierung von Ratten mit verschiedenen Antigenen, dass in den regionären Lymphknoten reichlich grosse, basophile Blasten hoher Teilungsaktivität auftraten, von denen bis zu 15% immunfluoreszenzoptisch nachweisbare, spezifische Antikörper und bis zu 30% Gammaglobuline enthielten. Weiter konnten dieselben in kinetischen Untersuchungen zeigen, dass aus diesen Blasten Plasmazellen und IgG-haltige Lymphozyten entstehen können. Auch in unserer Untersuchung liess sich in beiden in dieser Hinsicht ausgewerteten Fällen nachweisen, dass sich unter den DNS-synthetisierenden Lymphozyten auch IgG-positive Zellen befanden. Schliesslich konnten beim Durchmustern zahlreicher immunzytologischer Präparate auch vereinzelt fluores-

zierende Mitosen beobachtet werden. Bei den IgG positiven Zellen handelt es sich mit Wahrscheinlichkeit um solche, die zur Immunglobulinbildung und Sekretion befähigt sind [3].

Die unter der Einwirkung von Antigenen beobachteten Blasten stellen unter Immunisierungsbedingungen eine heterogene Zellgruppe dar, innerhalb derer nur ein Teil der D\N-Synthetisierenden Zellen als Vorstufen von Plasmazellen charakterisiert werden können. So waren in den oben erwähnten Untersuchungen von BAETZ *et al.* [6] Immunglobuline in der Mehrzahl der Blasten nicht nachweisbar. Auch wir fanden reichlich D\N-synthetisierende Zellen, die in der Immunfluoreszenz negativ waren. Ob es sich dabei um Zellen handelt, die die Fähigkeit zur Ig-Bildung noch nicht erreicht haben oder ob sie anderen lymphatischen Zellpopulationen angehören, kann aus der vorliegenden Untersuchung nicht geschlossen werden. Immerhin stellt die Mehrzahl der Blüthymphozyten Zellen dar, denen Entwicklungstendenzen zu Plasmazellen fehlen, die jedoch unter Einwirkung von Antigenen mitotisch aktiviert werden und an Reaktionen der zellulären Immunität beteiligt sein können.

Zusammenfassung

Bei 3 Patienten mit Rubellen und 3 Mäxernkranken bestimmten wir im Krankheitsverlauf mehrfach die Zahl IgG-haltiger sowie D\N-synthetisierender Lymphozyten mittels immunfluoreszenz- und autoradiographischer Technik. Bei den unersuchten Virusinfektionen waren beide Zellgruppen gegenüber den Normen deutlich vermehrt. Verlaufserhebungen ergaben einen gleichzeitigen Anstieg und Abfall beider untersuchter Zellpopulationen am Exanthembeginn. Überwogen jedoch die D\N-synthetisierenden Lymphozyten über die IgG-haltigen Zellen. Bei 2 Fällen fanden wir, dass sich ein kleiner Teil der IgG-positiven Zellen (14 und 1%) im Stadium der D\N-Synthese befand.

Wir danken Frau L. WERNER und Frau L. MUMMERS für die hervorragende technische Mitarbeit danken.

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Identification of Blood Monocytes by Demonstration of Lysozyme and Peroxidase Activity¹

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Abstract. By a simple cytochemical method for the demonstration of lysozyme (muramidase) activity more than 95% of human and rat blood monocytes could be identified and differentiated from lymphocytes. Peroxidase activity was seen in 83-88% of the monocytes. When a staining method for lysozyme and peroxidase activity in the same cell was used most blood mononuclear cells showed neither or both kinds of enzyme activity. Of the rat mononuclear cells 69% showed peroxidase activity alone.

Key Words:
Lysozyme in blood cells
Monocytes
Peroxidase staining
Rat blood

The identification of rat blood monocytes on the basis of morphological criteria has proved to be difficult [6-8]. A criterion clearly separating most or all monocytes from lymphocytes is, however, required for quantitative investigations of rat monocytes. In studies of monocyte kinetics rat blood monocytes have been identified as peroxidase-positive mononuclear cells [9-10].

RIKKA *et al.* [2] described an indirect cytochemical method for the demonstration of lysozyme activity in human blood and bone marrow cells. They observed lysis of *Micrococcus lysodeikticus* bacteria around monocytes, neutrophils, and some eosinophils, whereas no lysis was seen around lymphocytes.

The present study is based on the hypothesis that lysozyme activity as demonstrated by the technique of RIKKA *et al.* [2] is a criterion separating all monocytes from lymphocytes. The validity of this hypothesis is tested for human and rat blood.

¹ This investigation was supported by a grant from the S. and A. W. Foundation, Helsinki.

Materials and Methods

Materials Tail vein blood of 5 healthy male rats (aged 5–7 months) of an inbred Wistar strain was drawn and smeared for peroxidase staining. At the same time, 200–250 μ l of blood was collected into tubes containing EDTA for staining by other methods. Blood from the fingertips of 5 healthy humans was correspondingly smeared and collected.

Peroxidase staining The peroxidase staining technique described by RYTÖMÄÄ [5] was used. The reaction was rated as follows: – no staining of the cytoplasm, + yellow staining of the cytoplasm weaker than that of typical neutrophils, Yellow granules fewer than in neutrophils or present only in part of the cytoplasm, ++ the reaction of a typical neutrophil, i.e. intense yellow staining of the cytoplasm or yellow granules distributed all over the cytoplasm.

Demonstration of lysozyme activity The technique of BRIGGS *et al* [2] was slightly modified. 25 μ l of EDTA-anticoagulated blood and 25 μ l of a fresh suspension (60 mg in 1 ml of saline) of dried *Microroccus lysodeikticus* bacteria (obtained from Worthington Biochem. Corp.) were mixed in a tube, shaking gently for 5–10 s. A smear preparation was made from a drop of the mixture. The smears were allowed to dry in air and then fixed for 1 min in a 1:3 mixture of 10% neutral formalin and 96% ethanol. After rinsing in 0.01 M phosphate buffer (pH 7.0), the smears were incubated in this buffer for 10 min. After this they were air-dried and stained with May Grünwald Giemsa.

Lysozyme activity was seen as a zone of lysed bacteria surrounding the leukocyte. The groups of 4 bacteria were dissolved by disruption of the capsule, and the staining of the individual cocci was weaker than that of unaffected bacteria. Diminution of protoplast size was also seen. Rating of the reaction:

– no changes in the morphological characteristics of the bacteria immediately surrounding the cell, + part of the bacteria in immediate contact with the cell are lysed, ++ the cell is surrounded by a zone of lysed bacteria.

Demonstration of peroxidase and lysozyme activity in the same cell A mixture of blood and bacteria was smeared and fixed in the same way as for the demonstration of lysozyme activity. The preparations were incubated for 10–15 min in peroxidase reagent containing 0.05 M hydrogen peroxide and counterstained with May Grünwald Giemsa (Giemsa 5 min). In spite of light staining of the cytoplasm by May Grünwald Giemsa the yellow peroxidase reaction was readily seen.

When blood smears were stained for peroxidase, using 0.05 M hydrogen peroxide and an incubation time of 10 and 15 min, positive lymphocytes were not found in greater number than with the usual peroxidase staining. This speaks against the possibility of pseudoperoxidase reactions caused by the increased concentration of hydrogen peroxide and the prolonged incubation time.

Counting method The enzyme activity of typical monocytes, lymphocytes, and neutrophils was assessed by recording of the reactions of 500 cells of each type in human and rat blood. Only cells showing all the usual morphological characteristics of the cell type in question were considered as typical. The 500 cells comprised 100 cells from each individual.

Definition of monocytoid cells Mononuclear cells in rat blood were defined as monocytoid cells when they were not readily classified as either monocytes or lymphocytes on the basis of morphological criteria. The diameter of these cells was 10–15 μ m. The cyto-

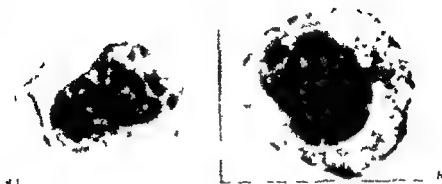


Fig. 1. Monocytoid cells of rat blood: *a* peroxidase-negative; *b* peroxidase-positive cell. Stained for peroxidase by the method of RATHMAA [9] and counterstained with hematoxylin ($\times 1375$).

plasm was relatively abundant. The nucleus was irregularly oval or bean-shaped and the chromatin pattern resembled that of monocytes or large lymphocytes (Fig. 1).

Results

Peroxidase reaction of blood leukocytes (table I). The peroxidase reaction of a typical monocyte in human blood consisted of light yellow staining of all or part of the cytoplasm and in rat blood of a few yellow granules unevenly distributed over the cytoplasm. Neutrophils and eosinophils were strongly positive while basophils were negative.

Lysosomal activity of blood leukocytes (table II). The lysosome activity of a typical monocyte was seen as a broad zone of lysed bacteria surrounding the cell. In rat blood the zone of lysed bacteria around monocytes usually was broader than that surrounding neutrophils (Fig. 2). Most eosinophils showed no activity. Basophils were not readily identified because of the background of fluorescent bacteria.

Combined lysosomal and peroxidase activity of blood mononuclear cells (table III). The great majority of the mononuclear cells fell into one of two groups: cells showing no activity ($1 - P$) and cells showing both kinds of activity ($1 + P+$). In rat blood, however, $9 \pm 1.4\%$ (mean \pm SD) of the mononuclear cells showed peroxidase activity alone ($1 - P+$). These cells were small mononuclear cells with a strong peroxidase reaction.

Table I Peroxidase activity of blood leukocytes Per cent of morphologically typical cells

		-	+	++	Cells counted
Human	Monocytes	12.4	62.2	25.4	500
	Lymphocytes	100.0	0.0	0.0	500
	Neutrophils	0.0	2.4	97.6	500
Rat	Monocytes	16.6	63.0	20.4	500
	Lymphocytes	99.0	1.0	0.0	500
	Neutrophils	0.0	6.6	93.4	500

Table II Lysozyme activity of blood leukocytes Per cent of morphologically typical cells

		-	+	++	Cells counted
Human	Monocytes	1.0	5.6	93.4	500
	Lymphocytes	100.0	0.0	0.0	500
	Neutrophils	0.2	0.2	99.6	500
Rat	Monocytes	3.6	7.4	89.0	500
	Lymphocytes	100.0	0.0	0.0	500
	Neutrophils	0.8	19.8	79.4	500

Table III Reactions of mononuclear blood cells stained for demonstration of lysozyme and peroxidase activity in the same cell Per cent of mononuclear cells

	L+, P+	L+, P-	L-, P+	L-, P-	Cells counted
Human	22.1	0.6	0.6	76.7	2 500
Rat	14.7	0.8	6.9	77.6	2 500

L = lysozyme activity P = peroxidase activity

Differential leukocyte counts of rat blood stained for lysozyme, peroxidase, and combined lysozyme and peroxidase activity To get an approximate idea of the proportion of positive mononuclear cells, blood was drawn from 5 rats and the blood of each rat was stained by the above mentioned 3 methods. It was found that $12.1 \pm 4.0\%$ (mean \pm SD) of the leukocytes were peroxidase-positive mononuclear cells. The mononuclear cells showing lysozyme activity

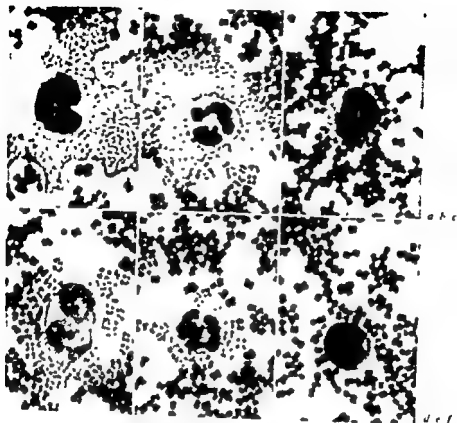


Fig. 2. Blood leukocytes stained for diaminobenzidine activity. A broad zone of lysed *Mycobacterium* bacteria is seen around a human monocyte (a) and a human lymphocyte (b). No morphological changes are seen in the bacteria surrounding the human lymphocyte (c) and the rat lymphocyte (e). The zone of lysed bacteria surrounding the rat monocyte (d) is characteristic, is narrower than that around the rat monocyte (d) ($\times 96$).

constituted $10.2 \pm 1.2\%$ of the leukocytes. With the combined staining $14.6 \pm 1.8\%$ of the leukocytes proved to be mononuclear cells showing either or both kinds of enzyme activity.

Number and enzyme activities of the monocytes. Differential leukocyte counts based on morphological criteria alone were performed on rat blood stained for peroxidase and counterstained with hematoxylin. In these preparations the cellular morphology was well preserved. Typical monocytes

constituted $10.7 \pm 3.2\%$ (mean \pm SD) and monocytoïd cells $8.9 \pm 2.6\%$ of the total of 4,100 leukocytes counted in 5 rats. Of 351 monocytoïd cells, 139 (37%) were peroxidase-positive.

The preparations made for demonstration of lysozyme activity did not offer as good a morphological detail as the peroxidase-stained ones. Thus, monocytoïd cells could not be reliably identified for the study of their lysozyme activity.

Discussion

The results show that lysozyme activity is a criterion separating nearly all typical human- and rat blood monocytes from lymphocytes. The specificity of the method of BRIGGS *et al* [2] has not been investigated, but it is probable that the agent causing lysis of the bacteria is lysozyme (muramidase) diffusing out of the cell. Attention has recently been focused on the monocyte lysozyme [1, 3, 4], and by an immunocytological technique lysozyme has been shown to be present in human monocytes, but not in lymphocytes [1].

Peroxidase activity was seen in rat blood not only in typical monocytes but also in many monocytoïd cells and even in a few small mononuclear cells classified as typical lymphocytes. This result is in agreement with the observations of WHITELAW [9].

The 6.9% of rat mononuclear cells which showed L-, P+ reaction were small. Since only 1.0% of the rat lymphocytes were peroxidase-positive, most of the L-, P+ cells must have belonged to the group of monocytoïd cells.

The majority of the monocytoïd cells did not show peroxidase activity. They thus resembled typical lymphocytes in this respect. Thirty-seven % of them resembled typical monocytes in being peroxidase-positive. Whether or not the monocytoïd cells or some part of them should be regarded as monocytes, cannot be decided before more is known of their functional characteristics.

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Ultrastructural Aspects of Bone Marrow and Peripheral Blood Cells in a Case of Plasma Cell Leukemia¹

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Abstract The submicroscopic morphology of blood and bone marrow elements in a patient with plasma cell leukemia Bence Jones proteinuria (type K) and hypogammaglobulinemia is described. Highly undifferentiated cells were rarely seen and tentatively called blast cells. Two other cell types with a more complex ultrastructural organization resemble the 'intermediate' lymphocytes and the plasmacytic elements and probably represent different functional stages of the same cell. In particular the absence of large polyribosomes and of dilated ergastoplasmic cisternae, and the presence of small polyribosome rosettes formed by 5-10 ribosomes, are correlated with the hypogammaglobulinemia and with the high L chain synthesis in this patient.

Key Words
Electron microscopy
Multiple myeloma
Plasmacytoma
Plasma cell leukemia
Ultrastructure in plasma cell leukemia

The plasma cell leukemia is a very rare form of immunocytic dyscrasia. Clinical course, laboratory findings, therapy and prognosis of this disease were recently discussed by PRUZANSKI *et al* [24] who reviewed 57 cases reported in the literature in the last 20 years. Furthermore, few studies concerning the ultrastructure of blood cells present in this type of leukemia have been published [1, 20].

The purpose of this paper is to describe the submicroscopic morphology of blood and bone marrow cells in a patient affected by plasma cell leukemia with Bence Jones proteinuria (type K) and hypogammaglobulinemia and to discuss the ultrastructural features of some cellular organelles in relation with the abnormal protein synthesis.

Case Report

A 65 year old man (M. G. No. 101) was admitted to the Institute of Medical Pathology of Milan (Italy) on April 1969 complaining of weakness, epistaxis and lower chest pain.

¹ This work was supported by a grant from CNR (N. 69 02171 0 115 2386).

started a few months before the hospitalization. The family history was noncontributory. Cardiac surgery had been performed because of diastolic ulcer at the age of 45. The patient appeared pale and severely ill. The physical examination revealed a tenderness over the last right rib, a hepatosplenomegaly but no lymphadenopathy. The erythrocyte sedimentation rate was 16 mm (Westergren) in 1 h. Hemoglobin 6.1 g%, red cells 1.937,000/mm³. The white blood cell count was 70,000/mm³ with 73% plasmacytes, 14% neutrophils, 7% monocytes, 1% promyelocytes, 2% myelocytes, 3% orthochromas, and erythroblasts. The bone marrow showed a diffuse infiltration by plasma cells (95% of the nucleated cells), many of which were highly immature and binucleated. Platelet count 30,000/mm³. Prethrombin activity 75%, fibrinogen 160 mg%. The Wassermann reaction was negative. Serum calcium 9.3 mg%, serum phosphorus 7.6 mg%, serum urea acid 10.6 mg%. Total bilirubin 1.3 mg%, alkaline phosphatase 37.8 mg Armstrong units/ml, glutamic oxalacetic transaminase 34.1 ml/g, glutamic pyruvic transaminase 18 U/ml. Blood urea nitrogen 61 mg%, plasma creatinine 1.46 mg%, glycerol triphosphate 10.6 ml/min. Urine contained protein in amounts up to 12 g/l. The Biuret Jones heat test was positive and the immunoelectrophoresis showed type A L chains. Serum proteins 4.2 g%, with 4.0 g% albumin and 1.4 g% globulin. Paper electrophoresis and immunoelectrophoresis of the serum showed an hypogammaglobulinemia of 0.15 g% and the presence of a type A Lence J-met protein. Cryoglobulin was not found and Coombs test was negative. Roentgenograms showed a diffuse micronodular infiltration in both lungs with several lesions in the last right rib, a diffuse osteoporosis of the pelvis and a columnar fracture of the thoracic vertebra.

The patient was treated with blood transfusions and butyric prednisone (40 mg daily) and methotrexate (4 mg daily). His condition slowly worsened and he died 24 days after admission with a clinical picture of pancreatic carcinoma (serum amylase 734 U%); firm evidence was lacking.

Methods

Three main preparations and peripheral blood cells obtained from the patient before operation and preoperative treatment were fixed with cold 2% glutaraldehyde [27] buffered at pH 7.3 [11] for 30 min. The samples were then fixed in phosphate buffer and then fixed for 1 h in 5% glutaraldehyde in cacodylate [11] and buffered in phosphate at pH 7.3. After at least 2 h of postfixation for 10 min the specimens were treated with cold 0.5% osmium tetroxide in cacodylate [10] at pH 5.5 for 10 to 30 min. Dehydration in methanol and propylene oxide and embedding in Epon 812 [10] were done following the procedure.

Thin sections were prepared with a Leitz ultrathin sectioning unit with a diamond knife, sectioned with a Leitz 10A and a Leitz 10B and then collected with a thin carbon film on a 300 mesh grid. The sections were stained with uranyl acetate and lead citrate and examined in a Zeiss 10A electron microscope at 100 kV.

Results

In the bone marrow and in the peripheral blood, plasma cells were present and at different stages of plasma cell development ranging from a 35-year



to typical plasma cells. Moreover, several nuclear and cytoplasmic anomalies like those described in multiple myeloma [8] were frequently observed.

At the ultrastructural level, this broad spectrum of cells was more evident, but their classification into distinctive morphologic categories was difficult. Nevertheless, it was possible to recognize various degrees or stages of ultrastructural organization considering the nucleolus size, the hetero/euchromatin ratio, the extension of the Golgi apparatus, the development of the rough endoplasmic reticulum, and the number of the polyribosomes.

The elements with the simplest submicroscopic organization are similar to the blast cells (fig. 1) and both in the bone marrow and in the circulating blood they are seen very rarely. In the large and rounded nucleus the finely dispersed chromatin is prevalent and the nucleolus is well evident. The relatively scarce cytoplasm contains many polysome rosettes, rare mitochondria, a little Golgi apparatus and a few profiles of rough endoplasmic reticulum.

Through transitional stages it is possible to observe 2 types of elements with a more extensive cytoplasm containing numerous organelles. In the first type (fig. 5) the nucleus is always eccentric and often kidney-shaped, the interchromatinic substance is abundant, the chromatin clumps are small and adjacent to the nuclear membrane and to the nucleolus. The main characteristic of the cytoplasm is the prominent Golgi apparatus mostly formed by vesicles and vacuoles which occupy a large portion of the cell (fig. 7). On the contrary the ribosomes, both arranged in polyosome clusters and associated with endoplasmic reticulum, are chiefly concentrated in the peripheral area (fig. 7). Numerous mitochondria, centrioles, osmophilic inclusions, and fibrillar formations can be seen (fig. 5).

The second type cells (fig. 6) have an ultrastructural organization similar to the plasma cell. The eccentric and irregular outlined nucleus has a very large nucleolus and numerous perichromatinic granules. Coarse chromatin aggregates are distributed in proximity of the nuclear membrane but also centrally. In the cytoplasm, the rough endoplasmic reticulum is prominent.

Fig. 1 Blast cell with a large and rounded nucleus with finely dispersed chromatin. The relatively scarce cytoplasm contains many polysome rosettes, rare mitochondria and rough endoplasmic reticulum profiles (peripheral blood $\times 10,500$).

Fig. 2, 3 Comparison between the ribosome organization in a normal plasmacyte (fig. 2) and in the elements of the plasma cell leukemia case (fig. 3). Note the simplest assembly of the ribosomes in figure 3 ($\times 137,000$).

Fig. 4 Two large perichromatinic granules surrounded by a light halo (arrow) are evident in the nucleus of a peripheral blood cell ($\times 37,000$).



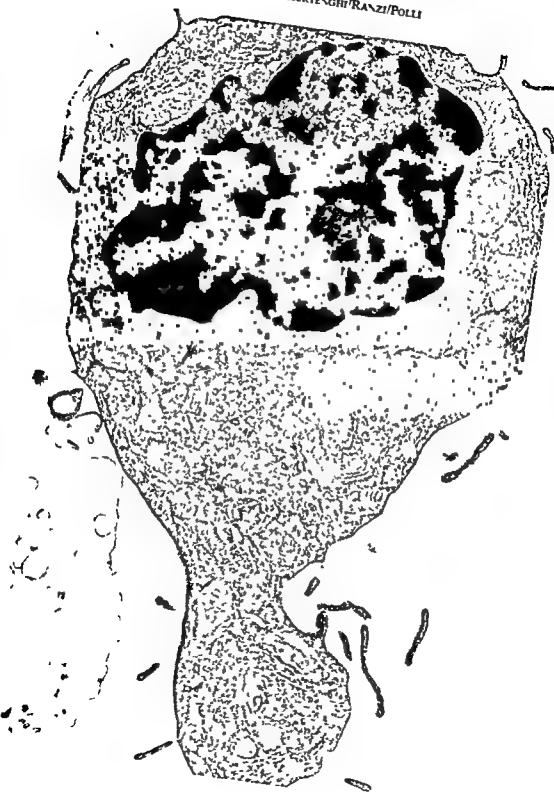
and represented by flattened and concentric cisternae which rarely appear dilated (fig 8). The Golgi zone is well evident. Osmiophilic inclusions and bundles of fibrils are frequently seen.

In spite of these different submicroscopic organizations, the described elements share some unusual features. 1. Some *perichromatinic granules* (fig 4) often have a greater size than normally (900-1 000 Å). 2. In several nuclei numerous *electron-opaque granular aggregates* bigger than interchromatinic granules can be seen (600-700 Å) (fig 12). 3. *Nuclear bodies* similar to those described in myeloma cells are rare (fig 11). 4. In many elements except blast like cells, the *nucleolus* has a rounded outline and assumes a 'target' configuration for the arrangement of concentric layers: the central region is a light area containing amorphous material without granules, the external layer is composed by a matrix of moderate density in which RNP particles are recognizable (fig 9). Sometimes these 2 zones are clearly separated by an intermediate ring of higher density which appears to be fibrillar (fig 10). Small clumps of intranuclear chromatin are frequently found. 5. In normal plasma cells the *polysome size* is different because of the variable number of ribosomes (fig 2). On the contrary, in every cell described here the *polysome size* is more constant and only small clusters of approximately 5-10 ribosomes are present (fig 3-5).

Discussion

The submicroscopic analysis of this plasma cell leukemia case allows to consider various ultrastructural problems about the immunocompetent cells. These do not represent a morphologically homogenous group: in fact, in experimental conditions, the elements synthesizing γ -globulins range from blast cells to lymphocyte like cells and to typical plasma cells [6, 13, 16]. Whether or not this variability demonstrated the derivation of the plasma cells from the lymphocyte remains to be determined. Nevertheless a similar broad morphologic spectrum was also described in the bone marrow of patients affected by multiple myeloma [8, 19].

Fig 5 First type cell. The nucleus is eccentric with small chromatinic clumps. The cytoplasm contains a large Golgi apparatus (G) while the polysomes and the rough endoplasmic reticulum lamellae are chiefly concentrated in the peripheral area. A centriole (arrow), some mitochondria and two osmiophilic inclusions (O) are evident (peripheral blood $\times 17 000$).



According to these data the bone marrow and peripheral elements in this case of plasma cell leukemia show various degree of ultrastructural organization. First of all rare highly undifferentiated cells were noted and tentatively called blast cells. They differ from phytohemagglutinin stimulated lymphocytes because of the absence of rough endoplasmic reticulum of pinocytosis vesicles of multivesicular vacuoles of electron-dense inclusions and because of the poor development of Golgi apparatus. It is also difficult to consider these cells as plasmoblasts because of the absence of ergastoplasm. On the contrary they resemble the hemocytoblast described by several authors [2-4].

The other cells with a more complex ultrastructural organization probably derive from these undifferentiated elements because transitional stages are noted. They can be considered more mature for the thick masses of chromatin clumps. In the first type cell the Golgi zone is prominent and the ergastoplasmic membranes are scarce compared with those of the plasma cells. They fairly resemble the intermediate lymphocytes described in cultures of phytohemagglutinin stimulated lymphocytes [15-16] and in the blood of patients affected by infectious mononucleosis [24-36] and by several diseases associated with elevated γ -globulin level [35-36]. Some authors [12-13-16] believe that these 'intermediate' lymphocytes synthesize antibodies.

Finally there are cells with well developed rough endoplasmic reticulum in the form of concentric lamellae. They more sharply resemble plasmacytic elements although they have a more dispersed chromatin, a big nucleolus and rarely dilated ergastoplasmic cisternae.

Both plasmacytic and lymphocyte like elements probably represent different functional stages of the same cell. In fact their classification was difficult because of the presence of cells with a transitional morphology.

The ultrastructural features of the cellular organelles permit other considerations.

Target nucleoli. The development of nucleolar light central area with loss of granules was observed in mature plasmacytes, lymphocytes and monocytes [30-31] in cultivated lymphocytes not yet stimulated by phytohemagglutinin [33] and in some cells after actinomycin D treatment [11-32].

Fig. 6. Second type cell. The eccentric nucleus contains a nucleolus and coarse chromatin aggregate. The rough endoplasmic reticulum is prominent and represented by flattened and concentric cisternae. The Golgi zone (G) is evident (bone marrow $\times 17,000$).



It is generally known that this drug inhibits RNA synthesis and it has been reported that in small mature lymphocytes of peripheral blood RNA and protein metabolism appear to be repressed [9-10]. According to these data the absence of granules in the central area of the so-called 'target' nucleoli might be an index of a diminished or anomalous production of nucleolar RNA.

Perichromatinic granules Attempts to determine the biochemical nature and functional significance of these structures have failed so far. MONSTERN *et al.* [22] found a modification of these particles in the liver cell after treatment with aflatoxine which inhibits RNA synthesis. Therefore the large perichromatinic granules in the described plasmacytic and lymphocyte-like cells might indicate a disturbed ribonucleoprotein synthesis.

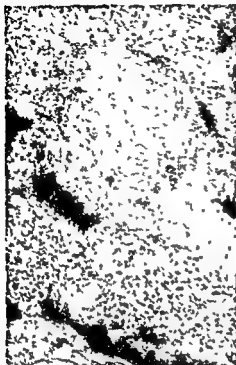
Intranuclear granule aggregates The nature of these inclusions is unknown, they are similar to interchromatinic granules but larger. Nevertheless similar aggregates have been observed in mouse plasmacytoma cells [3] and in human malignant lymphoma [17, 34].

Nuclear bodies They have been described in cells from numerous human tumors [14] and also in anomalous plasmacytes [5]. Recently SIVAR [28-29] demonstrated their increase in plasma cells after immunization and correlated their presence with an exalted protein synthesis.

Polyribosomes WILLIAMSON and ASKOVAS experiments [38] have indicated that in the mouse 5563 plasmacytoma heavy and light chains are separately synthesized by polyribosomes of different sizes. Heavy chains are formed by the large 300S polyribosomes approximately corresponding to 12-18 ribosome clusters [7]. On the contrary light chains are made by smaller 180S polyribosomes with 5-6 ribosomes. The subsequent assembly of light and heavy chains into IgG molecules occurs in ergastoplasmic cisternae which in the electron microscope appear dilated and containing flocculent material. In the described cells the large polyribosomes are absent and only small polyribosomes formed by 5-10 ribosomes are present. Besides the ergastoplasmic cisternae are very rarely dilated. These data can well correlate both with the hypogammaglobulinemia and with the high L chain synthesis in the patient. In fact the absence of large polyribosomes and of dilated ergasto-

Fig. 7. Particular of a first type cell. The prominent Golgi apparatus (G) and the small polyribosome clusters (arrows) are well evident (bone marrow $\times 21,000$).

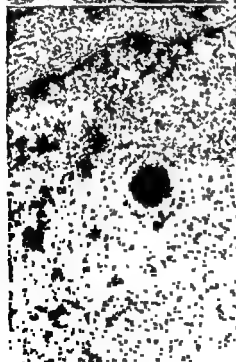
Fig. 8. Particular of a second type cell. The rough endoplasmic reticulum is well developed and concentric to the nucleus (n) (peripheral blood $\times 25,000$).



9



10



11



12

plasmic cisternae might indicate the incapacity of these cells to synthesize heavy chains and then complete γ -globulins.

To conclude, in this case of plasma cell leukemia the cell population is less homogeneous and presents a more pronounced degree of cellular organelle modifications in comparison with the multiple myeloma. This fact might be related to the more rapid clinical course of this rare immunocytic dyscrasia.

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Fig. 9. Target nucleolus in a first type cell. The central region contains an amorphous material without granules; the external layer is formed by a matrix with RNP particles. The nucleolus-associated chromatin is well evident (bone marrow, $\times 34,000$).

Fig. 10. Target nucleolus in a second type cell. The central and external zones are clearly separated by an intermediate ring of higher density (peripheral blood, $\times 34,000$).

Fig. 11. Nuclear body present in the nucleus of a second type cell (peripheral blood, $\times 25,000$).

Fig. 12. Electron opaque granular aggregate in a nucleus of a first type cell (bone marrow, $\times 64,000$).

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The Early Stages of Glass-Contact Haemolysis

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Abstract The morphological transformations of the early stages of glass-contact haemolysis of normal human red cells are described

Key Words

Erythrocyte deformation
Haemolysis by glass-contact

The purpose of this report is to provide the first complete description of the shape changes that precede contact haemolysis. In 1922, FENN [3] reported that red cells are haemolysed by contact with the surface of a clean glass slide. He established this by counting the number of cells in a microscopical field at different periods of time after addition of the suspensions to the chambers. This also determined, that the rate of contact haemolysis is influenced by the presence of serum: haemolysis occurred after a shorter period of contact in washed than in unwashed suspensions. FENN added, that he thought that the cells might assume a hemispherical shape before haemolysing. In 1938, PONDER and NEURATH [9] confirmed the view that the cells haemolyse in a hemispherical form. They did not, however, describe the transition from the biconcave disc to the hemisphere.

The transitional forms ranging from the biconcave disc to the hemisphere of contact haemolysis were identified during a reinvestigation of the disc/sphere transformation between slide and coverslip [1]. It was found, that two differing shape transformations occur when red cell suspensions are enclosed between slide and coverslip. One is the reversible disc/sphere transformation of freely floating cells in closed proximity to closely opposed ($<200\text{ }\mu\text{m}$ apart) glass surfaces. The other is associated with contact haemolysis. The shape changes preceding contact haemolysis were found to commence long before haemolysis occurs, and to become irreversible before the assumption of the hemispherical form. The two transformations have been inextricably

confused in the literature. In addition, many published photomicrographs indicate the widespread and unrecognised occurrence in current red cell research of the early stages of the haemolytic process. This has continued despite BESSIS' [2] repeated reference to one phase of the transition, and PONDEN's repeated reference to contact haemolysis.

Materials and Methods

Red cell suspensions were made from either peripheral or venous blood drawn from healthy donors. The blood drawn by venipuncture was defibrinated or had an anti-coagulant added. The anticoagulants used were ACD, EDTA, heparine and citrate. Unwashed cell suspensions were prepared by adding 0.02 ml of blood to 5 ml of a suspension fluid. The suspension fluids used were (a) 1% NaCl, (b) a pH 6.9 mixture (4:1) of 1% NaCl and 0.111 M Schrenken's phosphate buffer [4], and (c) pH 7.3 Michael's veronal acetate buffer [5]. Washed cell suspensions were prepared from the unwashed cell suspensions. The unwashed cell suspensions were centrifuged, and the supernatants were aspirated off. The cells were then washed 3 times. The procedure for washing the cells was to resuspend them in a fresh 10-ml portion of an appropriate suspension fluid. This was followed by centrifugation and aspiration of the supernatant fluid. After the third washing the cells were resuspended in a fresh 5-ml portion of an appropriate suspension fluid.

The American Hospital Supply slides and coverslips were made from extremely hard glass with low alkali content, and the slides were pre-cleaned. Both the slides and coverslips were cleaned before use. They were soaked in haemo-sol detergent from 2 to 24 h, rinsed in 5 changes of hot tap water and then 3 changes of glass-distilled water and air dried. The slides and coverslips were manipulated in pyrex carriers during this procedure and the faces that were to come in contact with the suspensions were not touched when the microscopical preparations were made.

The microscopical preparations were made by placing 2 or 3 drops of suspensions on a slide and then putting a coverslip on the drops. The edges of the coverslips were coated with vaseline before they were placed on the drops. This sealed the preparations, and helped to ensure that the glass surfaces were more than 200 μ m apart. The preparations were observed with light and phase-contrast microscopy.

Results and Discussion

The observations were unaffected by either the method of collecting the blood or the suspension fluids used.

Washed red cells started to undergo subtle dynamic shape transformations shortly after they settled on the slide. Within 5 min of the making of the microscopical preparations, those discs which had settled and were lying flat on the slides showed rapid movements along their outer edges. These

appeared as many exceedingly shallow waves. The rapidity of their formation and disappearance, coupled with their small amplitude, made it impossible to determine the length of these waves, or to establish whether they ran into each other. They did not, however, seem to be organised in any general pattern. No changes could be detected over the top of the rim, nor along the inner edge of the rim, nor over the biconcavity.

Ten min after the washed cell suspensions had entered the chambers, the cells which had settled down first displayed an easily recognisable and distinctive static phase of the pre-contact-haemolysis shape change. There were still no detectable changes over the top of the rim, nor along its inner edge, nor over the concavity. The outer edge of the cell, however, was decidedly serrated. These serrations were all in the plane of contact of the cell with the glass, and had sharp points and broad bases, i.e. they were much wider than they were high [10]. The bases were joined to each other by such smooth curves, that no trace remained of the original circular edge. The shape of these serrations suggested, that they were as much the result of retraction of parts of the cells, as projections from the cells. It is impossible to decide between these alternatives by measuring the diameters of the cells, because the edges are too irregular for such measurements. The density and phase-contrast halo of these cells, but they lost the shimmer or flicker that is seen in free-floating cells viewed with phase-contrast microscopy [12]. The form persisted from 10 to more than 30 min from the time of its first appearance.

These serrated concave discs changed, in 7–9 s, into prolytic hemispheres. This was accomplished by the cells simultaneously regaining a circular circumference, which extended beyond the points of the serrations, and losing their concavity. This change was not smooth, it was accompanied by sudden violent distortions of the cell. This form had a larger diameter than the original disc, and had the dusky appearance common to prolytic spheres [6]. In phase-contrast microscopy, the dusky appearance is associated with a decrease in density and a smaller halo around the cell. The duration of this form also varied from 10 to more than 30 min. Haemolysis of the prolytic hemispheres occurred in 2–3 s without any further shape changes. The ghosts underwent the deterioration of ghosts stuck to glass or plastic as described by PONDER and BARRETO [8].

Unwashed cell suspensions followed the same course as washed cell suspensions, but the process began 20 min later.

The coverslips were tapped to set up currents in the preparations in an effort to dislodge the cells from the slides. All of the hemispherical cells

haemolysed in that form without being dislodged from the glass surface. The serrated concave discoids could be divided into 2 groups. The cells in one group appeared more rigid than the normal cell, i.e. they were not distorted in the fluid currents, and freely floating biconcave discs bent around them. Moreover, those that were successfully dislodged persisted as crenated discoids with the dusky appearance. Others of this group that were not dislodged, haemolysed as serrated discoids, and their ghosts retained this form for a short while. These ghosts also eventually underwent spontaneous contractions [8]. The second group of serrated discoids were earlier stages of this form. They showed the usual liquid drop-like distortions of cells stuck at 1 or 2 points to a slide. When these cells were finally floated free, they regained the normal biconcave form.

The addition of plasma or serum to preparations containing freed dusky-crenated discoids caused no changes in these forms. It seems therefore, that these are the irreversible forms observed by FURCHGOTT [4] and PONDRA [7] in glassball treated cell suspensions, and they are prolytic. Finally, it is apparent that TRITEL-BERNARD found the rigid serrated form birefringent [11]. Thus these serrations are, like type II crenations, associated with local orientation of haemoglobin, and a diminution of PONDRA's R [6].

Little more can be said as to the cause of these changes other than that they result from a spreading of the cell on the surface of a slide. But it is important to recognise that 10 min after a slide and coverslip preparation has been made many of the normal red cells are changed by contact with the glass surface and, in some instance, these alterations are irreversible.

Acknowledgement The author wishes to thank Prof R G MACFARLANE for his assistance in the preparation of this report.

This work was supported in part by grants from the New York and British Heart Foundations, and D M BARRETO was a Post Doctoral Fellow, USA NIH.

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The Quantitative Bone Marrow and Spleen Composition in Male NMRI and CBA Mice

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Abstract The splenogram, myelogram and quantitative data on the bone marrow and spleen of 2 strains of mice are presented. Using 2 techniques, the absolute numbers of different cells per mg of bone marrow and per femur are determined. The results are discussed with respect to the quantitative evaluation of the whole body haemopoietic tissue in mice.

Key Words
Haemopoiesis in animals
Mouse bone marrow
Mouse spleen

Mice as experimental animals are used under widely varying conditions where reactions of the haematopoiesis occur and are of interest. In spite of this only a limited number of references, relating to the normal bone marrow, are available [2, 15, 20]. The references quoted for the morphology of the bone marrow cells often fail to present quantitative data [2, 15] and vice versa [11, 13] and no data seems to be available on the spleens of these animals. In this paper the results of the qualitative and quantitative evaluation of the whole haemopoietic system in 2 strains of mice used for radiobiological studies in our laboratory are reported. For the quantitative evaluation, 2 different techniques are used and the results from both are compared. As the recognition of the different cell types in the bone marrow presents considerable difficulties, their morphology is described.

Material and Methods

Male mice of the 2 strains were studied. The commercially bred NMRI albino mice were between 4 and 6 weeks old and weighed 25-32 g. The CBA mice obtained from the Institute of Genetics, University of Stockholm, were 8 weeks old and weighed 25 g. The

mice were caged in the same room and were fed on standard pellets and water provided *ad libitum*

For the bone marrow and spleen studies, the mice were killed by cervical fracture. As in preliminary studies, neither qualitative nor quantitative differences were found between the cell populations of the right and left femur, the left femur was used for differential counts and the determination of the number of cells per mg of tissue, the right femur was used for the determination of the total number of cells per whole femur.

In determination of the iron uptake of the bone marrow, ^{59}Fe was used. The mice were injected intraperitoneally with $0.1 \mu\text{Ci } ^{59}\text{Fe}$ (ferric citrate, 25 mCi/mg), in 0.2 ml saline and killed 5 h later.

Determination of the number of marrow cells per mg of tissue The modified method of PO-CHUEN CHAN *et al* [17] was used. Each femur was split longitudinally, the marrow was aspirated into preweighed heparinized microcapillary tubes and reweighed to determine the net marrow mass. After expelling the marrow into a tube containing a known amount of Hank's medium, the ^{59}Fe activity was determined as a percentage of the injected activity calculated per 1 mg. A homogenous suspension was then made using a fine Pasteur pipette and the total number of nucleated cells in the suspension was determined using a haemocytometer or cell counter (Celloscope 302, Ljungberg, Sweden). In the latter case, cetrimide was used for erythrocyte lysis [16] and the aperture current and amplification were fixed in order to discriminate between a volume range from 25 to $750 \mu\text{m}^3$, thus avoiding counting of thrombocytes and cell debris, but permitting all nucleated cells to be counted. The number of nucleated cells per mg was calculated. After centrifuging the cell suspension, the diluent was removed, the packed cells were resuspended with calf serum and multiple smear preparations were made. The smears were stained with May Grünwald Giemsa stain at a controlled pH of 4.5 which gave the best staining results. Two stained smears were selected for the differential counts, 500 cells were evaluated in each slide and the number of each cell type was calculated per mg of bone marrow. Damaged cells representing about 10–15% of the cell population were not included in the differential counts. There is no evidence, perhaps with the exception of reticular cells, that certain types of the 3 main cell lines are more easily damaged than others during the smear procedure and thus the number of damaged cells was assumed to be randomly distributed among the marrow cell types. This is supported by the evidence that in spite of profound changes in the marrow cell composition after the cessation of hypoxia [1] the relative number of damaged cells was approximately the same.

Determination of the number of marrow cells per femur The femur was cleaned carefully from the surrounding tissue and the ^{59}Fe activity was measured. Then, the proximal epiphysis was cut at a standard point (the proximal end of the trochanter tertius) and the ^{59}Fe activity of the shaft was determined again. The bone marrow was expelled into the Hank's medium, and the total number of different cell types per femur shaft was determined using the same procedure as mentioned above. From this, assuming the ratio between the number of cells per shaft and femur to be the same as the ratio between the ^{59}Fe activities, the number of nucleated cells per femur was calculated.

Examination of spleen The spleen was removed, weighed and placed in Hank's solution. Cell suspensions were made by gently chopping and teasing of the tissue and by repeated aspiration through a fine Pasteur pipette. The suspension was then allowed to sediment for a standard time of 1 min for elimination of the stroma, then the supernatant

was carefully removed and repeatedly aspirated through a fine needle. The evaluation of the spleen was the same as for the bone marrow. Because in the spleen it was the erythropoiesis and the myelopoiesis which were of primary interest, the morphological classification of the other cell types has not been carried out in detail.

Classification of bone marrow and spleen cells. For mouse haemopoietic cells it is not possible to apply the criteria routinely used for human cells. The erythroid cells retain the cytoplasmic basophilia later than the corresponding cells in man and thus is it inappropriate to classify normoblasts as baso-, poly-, and orthochromatic. Therefore, classification was based on the nuclear structure and cell size and the alternative classification of early, intermediate and late normoblasts [9] were used. Erythroblasts (proerythroblasts) were somewhat smaller than myeloblasts ($11.0\ \mu\text{m}$). They were usually round with a finer nuclear structure and more cytoplasmic basophilia. There was frequently a pale halo near the nucleus. Early normoblasts correspond in size ($8.6\ \mu\text{m}$) and morphology to those of man and higher mammals. Intermediate normoblasts had a persistent high degree of cytoplasmic basophilia. These cells were smaller in size ($6.3\ \mu\text{m}$) than the early normoblasts, but the nuclear morphology was very similar except that larger blocks of chromatin were present. Late normoblasts ($4.5\text{--}5\ \mu\text{m}$) were, in our preparations, very often cells with basophilic cytoplasm. The nuclei contained either highly condensed chromatin or were pyknotic.

Myeloid cells. The scarcity or complete absence of cytoplasmic neutrophil granules and the maturation of granulocytes by formation of ring nuclei [7], rather than by the characteristic metamyelocyte-stab cell sequence complicate the identification of cells. In the case of rats, it was assumed [26] that these ring forms were intermediate, between the myelocyte and metamyelocyte stage. According to our observations, which agree with those of HULTZ [7], the central hole in the nucleus is not confined to one particular stage of maturation, and appears first at the promyelocyte stage.

Myeloblasts were cells, having round or somewhat irregular nuclei with fine chromatin and recognisable nucleoli without a central hole and sparse basophilic cytoplasm containing no granules. The mean diameter was $13.5\ \mu\text{m}$. Promyelocytes were cells with a mean diameter of $12.5\ \mu\text{m}$ in which the chromatin was somewhat coarser and which in some cases contained a few eosinophilic granula in the cytoplasm. Nucleoli were less apparent and a very tiny central hole was sometimes present in the nucleus. Myelocytes formed the most

numerous and a relatively high nuclear-cytoplasmic ratio. In the first case, the nucleus was round or slightly oval, eccentrically situated. If the central hole was present in the nucleus, the cell was classified as a myelocyte, when the hole diameter did not exceed approximately $1/3$ of the total nuclear diameter, having smooth nuclear borders and a basophilic cytoplasm. The cell diameter varies considerably with a mean of $10.9\ \mu\text{m}$.

Myelocytes were cells with a mean diameter of $10.9\ \mu\text{m}$ in which the chromatin was somewhat coarser and which in some cases contained a few eosinophilic granula in the cytoplasm. Nucleoli were less apparent and a very tiny central hole was sometimes present in the nucleus. Myelocytes formed the most

ing to the nuclear morphology, they were classified as eosinophil myelocytes (mean diameter $12.7\ \mu\text{m}$) or eosinophils (mean diameter $10.1\ \mu\text{m}$)

Lymphoid cells For the evaluation of lymphoid cells in the bone marrow YORREY's classification [28] was used with small exceptions. Undifferentiated cells with basophilic cytoplasm but rather dense pachychromatic nucleus and mean cell size about $9\text{--}10\ \mu\text{m}$, not classifiable as transitional cells, were termed 'basophile mononuclears'. In the spleen, the term large lymphocytes and not transitional cells was used. All blasts not clearly classifiable as erythro- or myeloblasts were grouped as lymphoblasts.

The picture is rather complicated, and it is necessary to achieve preparation of smears with even distribution of cells sufficiently spread out, and accurately stained, so that nuclear details are in evidence.

Results

For the quantitative evaluation of the bone marrow, 2 techniques were used, based either on the estimation of the marrow cellularity per mg of tissue or per whole femur. Both results are summarized in table I. For the differential counts and quantitative estimation of the cellularity per mg, the left femur was used and the studies were made with $3.2 \pm 0.1\ \text{mg}$ of bone marrow in CBA mice and $2.6 \pm 0.2\ \text{mg}$ in NMRI mice. Using radioactive iron, the radioactivity for the femur shaft was found to be $78.8 \pm 4.5\%$ of the identical whole femur radioactivity and this ratio was assumed also for the marrow cellularity. Both techniques were quantitatively compared using the correlation between the radioactivity per mg of the bone marrow and the whole femur activity corrected for the activity of the bone only. Carefully cleaned from marrow cells, the femur bone still contains $14 \pm 2\%$ of the whole femur activity. The amount of bone marrow in one femur was determined in this way in 6 CBA mice to be $10.5 \pm 0.6\ \text{mg}$, and the ratio between the number of cells per 1 mg and 1 femur about 1:10, which fit well with the results presented in table I. In the bone marrow differential counts, the erythroid part is significantly lower in the CBA mice and this difference is still seen if the number of erythroid cells per mg is used as criterium, because there was no significant difference in the number of nucleated cells per mg of tissue. However, when the results are expressed per femur, the difference becomes smaller (table I).

The differential count and the quantitative data on the spleen are presented in table II. The total number of nucleated cells recovered per spleen is significantly higher in the CBA mice and the lymphoid and myeloid part of the spleen are responsible for this. The total number of erythroid cells in the CBA mice is somewhat lower than in NMRI.

Table 1. The myelogram and the absolute number ($\times 10^6$) of cell types per mm³ of bone marrow and per one femur in male NMRI ($n=14$) and CBA ($n=6$) mice. The mean and the standard error of the mean are given. 1 000 cells were counted. The undifferentiated cells include a few histiocyte-like cells, plasma cells, reticular and endothelial cells

	NMRI				CBA							
	Myelogram		Cells/femur ($\times 10^6$)		Myelogram		Cells/femur ($\times 10^6$)		Myelogram		Cells/femur ($\times 10^6$)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Erythroblasts	0.4	0.1	11.3	3.9	0.09	0.02	0.3	0.2	7.0	4.2	0.10	0.05
Early normoblasts	1.3	0.2	31.1	8.1	0.26	0.03	0.4	0.2	9.9	4.8	0.10	0.05
Intermediate normoblasts	5.2	0.5	122.3	17.0	3.05	0.10	1.6	0.7	39.4	19.8	0.40	0.19
Late normoblasts	9.8	1.0	230.3	31.9	2.00	0.31	4.7	1.8	116.3	47.7	1.22	0.48
Total erythroid cells	17.0	2.0	409.0	57.9	3.48	0.34	7.2	2.5	174.5	73.2	1.83	0.71
Myeloblasts	0.7	0.1	15.9	2.7	0.14	0.02	0.8	0.1	18.4	3.1	0.19	0.03
Proneurocytes	1.6	0.1	35.8	3.3	0.31	0.02	2.8	0.8	70.4	13.4	0.72	0.12
Myelocytes	4.5	0.4	98.7	7.6	0.91	0.08	0.9	1.2	223.1	46.0	2.29	0.39
Metamyelocytes	7.3	0.6	167.9	23.0	1.49	0.12	11.3	1.1	273.7	42.8	2.88	0.42
Neutrophils	30.3	2.4	697.4	69.1	6.23	0.48	20.3	1.3	506.1	106.5	5.24	0.74
Eosinophil myelocytes	0.6	0.1	14.3	4.4	0.13	0.02	1.7	1.0	45.9	12.8	0.45	0.11
Basophils	2.7	0.2	64.6	6.3	0.56	0.03	2.4	0.3	61.2	10.8	0.62	0.09
Total myeloid cells	47.8	2.7	1,093.3	99.7	9.77	0.55	46.7	3.0	1,193.4	183.8	12.32	1.64
Small lymphocytes	27.1	2.3	575.9	57.2	5.53	0.37	33.4	2.9	832.0	17.8	8.76	0.55
Transitional cells	6.3	0.8	154.6	22.2	1.28	0.16	5.5	0.5	137.2	21.2	1.43	0.21
Basophil mononuclears	0.7	0.1	16.7	4.3	0.14	0.02	0.5	0.1	10.9	4.4	0.13	0.04
Lymphoblasts and undifferentiated blast	1.6	0.2	39.1	7.1	0.32	0.04	1.8	0.2	43.5	5.6	0.44	0.04
Total lymphoid cells	35.6	1.9	781.6	73.3	7.28	0.39	43.2	3.6	1,025.2	49.0	10.76	0.82
Megakaryocytes	0.22	0.18	5.5	1.3	0.04	0.01	0.1	0.05	2.8	1.5	0.03	0.01
Undifferentiated cells	0.6	0.1	13.5	3.6	0.11	0.02	1.34	0.4	36.9	9.7	0.40	0.09
Mitotic cells	1.0	0.1	23.4	2.7	0.19	0.02	0.4	0.1	9.1	2.1	0.05	0.02
Nucleated cells, mg of bone marrow $\times 10^6$			2,279	1,332			2,436					
Nucleated cells, femur $\times 10^6$			20.44	1.52			23.30					

Table 11 The splenograms and the absolute number ($\times 10^6$) of cell types per spleen in male NMRI and CBA mice. The mean and the standard error of the mean are given. One thousand cells were counted

	NMRI			CBA		
	Splenogram		Cells/spleen ($\times 10^6$)	Splenogram		Cells/spleen ($\times 10^6$)
	Mean	SE		Mean	SE	
Erythroblasts	0.08	0.04	0.11	0.07	0.07	0
Early normoblasts	0.7	0.2	1.01	0.30	0	0
Intermediate normoblasts	2.7	0.9	4.10	1.40	0.03	0.76
Late normoblasts	5.2	1.1	9.39	3.01	1.63	8.76
Total erythroid cells	8.6	2.1	14.56	4.57	1.6	9.64
Myeloblasts	0.07	0.04	0.10	0.07	0	0
Promyelocytes	0.12	0.07	0.22	0.14	0.5	0.23
Myelocytes	1.1	0.3	2.15	0.80	0.2	5.23
Metamyelocytes	1.2	0.3	1.97	0.54	0.4	8.00
Neutrophils	1.6	0.3	2.66	0.51	1.4	12.48
Lysinophil myelocytes	0		0	0.05	0.04	0.18
Eosinophils	0.8	0.6	0.32	0.14	0.04	0.30
Total myeloid cells	4.3	0.6	7.45	1.54	0.9	25.11
Small lymphocytes	69.8	3.6	134.46	33.61	1.1	228.05
Large lymphocytes	14.1	1.6	23.43	4.59	0.7	59.38
Lymphoblasts	0.9	0.3	1.04	0.25	0.2	4.22
Basophil mononuclears	0.6	0.1	1.22	0.33	0.2	3.59
Histioid cells	0.7	0.2	1.58	0.66	0.2	5.10
Plasma cells	0.5	0.1	1.20	0.40	0.2	4.04
Mitotic cells	0.4	0.2	0.92	0.50	0.02	0.06
Undifferentiated cells	0.2	0.1	0.30	0.15	0.2	2.89
Total lymphoid cells	86.9	2.4	163.55	38.50	2.5	300.75
Nucleated cells/spleen $\times 10^6$	-	-	186.00	43.40	-	347.70
						16.10

Discussion

There appear to be 3 main factors accounting for the differences in the bone marrow picture reported in the literature. The first is the difficulty associated with the classification of mouse bone marrow cells. There are no differences regarding the erythroid and myeloid series, but the cells of the lymphoid complex are grouped in rather different ways in different reports. The second factor is correction for the numbers of damaged cells which in most instances, depending on the techniques used, constitute about 10-20% of nucleated cells. The third factor is the differences between the strains. On account of these factors exact comparison is very difficult. The main point appears to be the different relationships between the erythroid and the lymphoid parts of the bone marrow, according to the strains used in the different investigations [2, 15, 19]. However, comparison of the quantitative data is still more difficult even if the same mouse strain is used in different experiments. For quantitative marrow studies principally 2 different techniques have been used in the mice. The first involves the counting of cells washed out from the bone marrow cavity after the ends of the bones had been cut off and the number of cells calculated per femur shaft or per unit length of diaphysis [4, 11, 13, 25]. The second is based on measurement of the cellularity per mg of marrow [17] or per mm³ of marrow [14]. The validity of the determination per mg of tissue is based on some assumptions discussed earlier by others [17]. In spite of the small amount of bone marrow used, the results are fairly reproducible and the values obtained in our studies on NMRI albino mice are in very good agreement with those reported by PO-CHEU CHAN *et al* [17] on Swiss mice. The data obtained in the CBA mice differ in some respects regarding the different proportions of the cell types. However, using the determination of the cellularity per mm³, PROO [14] reported values of 2.41×10^6 cells for CBA mice, which nearly correspond to our results for the same mouse strain.

It is not possible to compare the results of the techniques which use the determination of the cells per femur shaft, if the relation to the cellularity of the total femur is not specified and the calculation is made per unit length. To eliminate these difficulties, the measurement of the ⁵⁹Fe activity in the whole and in different parts of the femur should be used to define the parameters.

In the mouse, splenic erythropoiesis was recognized as the characteristic feature of the normal state [8]. In special situations the spleen is capable of greatly increasing the erythropoietic activity [1, 3, 10, 12, 18, 24] and this

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Proliferation Disturbances of Erythroblasts in Congenital Dyserythropoietic Anemia Type I and II¹

W. QUTScher, E. SPIERTZ, E. JOST and H. HEIMPEL

Abstract DNA synthesis of erythroblasts in congenital dyserythropoietic anemia (CDA) type I and II was studied. In CDA type I, hypertetraploid and octaploid cells are present in all stages of maturation. In polychromatic normoblasts, only a few labelled cells are observed and the majority of cells show DNA values in the pre-tetraploid range. The results suggest a disturbance of mitotic division which becomes apparent already in the basophilic stage, and an arrest of DNA synthesis in the more mature nucleated red cells, dependent from cytoplasmic hemoglobin concentration. In CDA type II, a shift of the G₁/S ratio was observed in mononucleated and binucleated polychromatic cells consistent with repression of DNA synthesis-induction earlier than normal. The binucleated cells are probably the result of normal karyokinesis not followed by cytoplasmic division.

Key Words

Autoradiography
Congenital dyserythropoietic anemia
Cytophotometry
DNA synthesis
Erythropoiesis
Hereditary refractory anemia

The term 'congenital dyserythropoietic anemia' (CDA) has been introduced for a group of hereditary refractory anemias, which are characterized by ineffective erythropoiesis, specific cytopathology of the nucleated red cells in the bone marrow and secondary hemochromatosis [2, 12]. A subclassification of CDA into 3 types according to the different morphological abnormalities of the nucleated red cells was proposed by HEIMPEL and WEEDT [6] and has been supported by membrane abnormalities of the peripheral red cells in patients with CDA type II, but not with type I [3, 4]. In order to elucidate the nature of the abnormal structure of the erythroblasts in CDA type I and II, the bone marrow has been investigated by a combined cytophotometric and autoradiographic technique using tritiated thymidine

¹ Supported by Deutsche Forschungsgemeinschaft

(^3H TdR) *in vitro* This method which has been extensively used for the study of ineffective erythropoiesis [1] may be suitable for the detection of proliferation disturbances in this disease

Methods

Patients History, physical findings and the results of laboratory investigations of 5 of the 6 patients have been described in detail in a previous paper [7]. For this reason only the most important hematological data at the time of the bone marrow aspiration will be represented (table I). In table I the case numbers of the previous publication are given. The marrow of case 6¹ showed identical cytopathology of the other 2 cases with CDA type II.

General procedure The marrow was aspirated into a syringe containing 0.5 ml EDTA solution (1% Na_2EDTA in 0.7% NaCl), and incubated for 1 h with ^3H TdR (concentration $\equiv \mu\text{Ci/ml}$, specific activity 2.0 Ci/mv) at room temperature. Smears were made from the marrow spicules and stained with Pappenheim stain. The individual cells were marked with an object marker and photographed for subsequent localization for consecutive cytophotometry and autoradiography, and for the determination of the nuclear size by planimetry. Upon completion of the photography, Pappenheim stain was leached out by treatment with 50% ethanol for 5–10 min. The smears were restained by the Feulgen method with hydrolysis for 12 min in 1 N HCl at 60°C. The modification of GRAUMANN [5] was used applying pararosanilin for Schiff's reagent. The staining time was 45 min.

Cytophotometry For the cytophotometric determination of the DNA content a MPV-cytophotometer (Leitz, Germany) was used. Monochromatic light at 370 nm was employed with the size of the photometric field being $0.42 \mu\text{m}^2$. The extinction was measured at 10 or 20 points within the nucleus. The relative DNA content (AU=arbitrary units) of individual nuclei was calculated from $\text{AU} = E \times A$ (E =extinction, A =nuclear area). The nuclear areas were determined by planimetry (cases 1–3) or calculated from the nuclear diameters (cases 4–6).

Autoradiography - Autoradiograms of the Feulgen stained smears were made by the dipping film technique using the Ilford L4 liquid emulsion. The smears were exposed for 25–35 days. The background was estimated by counting the grains within lymphocyte nuclei. Cells with more than 3.5 grains were designated as labelled.

Results

Determination of the diploid standard For evaluation of the diploid standard (2c) the DNA content of about 50 bone marrow lymphocytes was measured. The arithmetic mean was calculated and compared with the

¹ The bone marrow of this case could be investigated by the courtesy of Prof. SCHUBOTHE, Medizinische Universitätsklinik, Freiburg.

Table I Main hematological values of the patients

Diagnosis	Nr	Case	Hb g/100 ml	RBC 10 ⁶ /μl	Wk, %	MCH, pg	MCV, μm ³	Retc, %	WBC/mm ³	Platelets/ mm ³	Total bilirubin, mg%
CDA I	1 (1)	C.B., female born 1949	9.4	2.7	33	35	122	28	4,000	219,000	1.80
	2 (3)	F.J., female born 1928	9.2	2.4	29	33	120	26	9,500	610,000	2.35
	3 (4)	K.W., male born 1922	10.2	3.2	35	34	112	11	9,100	404,000	0.91
CDA II	4 (5)	K.H., male born 1930	10.6	3.7	27	29	73	61	7,200	193,000	2.25
	5 (6)	H.J., male born 1948	8.8	2.9	26	34	87	8	6,500	356,000	1.62
	6	F.V., male born 1937	11.0	3.7	30	30	81	79	4,900	110,000	3.18

In parentheses case numbers of reference 7

Table II Comparison of the mean relative DNA content of lymphocytes and G₁-erythroblasts

Case number	Mean DNA of lymphocytes (2c)	Mean DNA of G ₁ -erythroblasts	Difference, %
1	30 ± 0.5 ¹	28 ± 0.7	- 7.9
2	30 ± 0.6	43 ± 0.6	-10.6
3	49 ± 0.3	47 ± 0.4	- 4.5
4	10.5 ± 2.1	10.2 ± 1.7	- 4.1
5	11.1 ± 1.2	10.6 ± 1.3	- 4.2
6	12.2 ± 1.7	11.6 ± 1.9	- 4.5

¹ Standard deviation

mean diploid values, which were determined from the unlabelled diploid basophilic erythroblasts (cases 1-3) and the diploid basophilic and polychromatic erythroblasts (cases 4-6) representing G₁ (table II). The results demonstrate that the diploid standard in G₁-erythroblasts is 4.1-10.6% lower than in the lymphocytes.

DNA content and DNA synthesis stages in CDA type I Mononucleated cells. The results of the study of case 2 are given in figure 1. The following

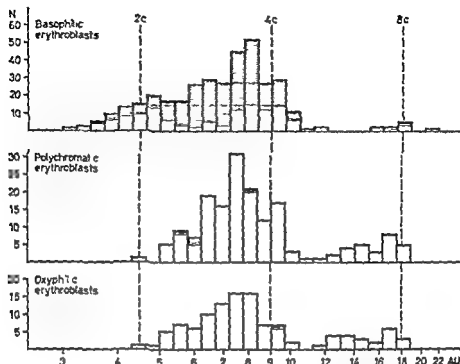


Fig 1 Relative DNA content in arbitrary units (AU) and ^3H TdR labelling (shaded areas) of mononucleated erythroblasts in CDA type I (case 2). Semilogarithmic scale. N Number of cells. 2c Diploid standard evaluated from basophilic G_1 -erythroblasts. 4c Tetraploid DNA content. 8c Octoploid DNA content. For numbers of cells measured see table III. In the basophilic compartment at least 100 large, 100 intermediate and 100 small basophilic erythroblasts were counted.

stages of the cell cycle can be distinguished by the method used: the diploid cells not labelled with ^3H -TdR represent the post mitotic resting period (G_1), the tetraploid and not labelled cells the pre-mitotic resting period (G_2), and the labelled cells the DNA synthesis period (S).

As seen in figure 1, a normal cell cycle is observed only in the basophilic erythroblasts showing a number of cells in G_1 , S and G_2 . In the polychromatic compartment only a few cells are labelled. The distribution of the DNA content is highly pathological in all cell types. An appreciable number of hypertetraploid and octoploid cells is present. The majority of the polychromatic as well as of the oxyphile cells shows DNA values within the pretetraploid range, and diploid cells are rare.

The data related to the above mentioned stages of the cell cycle in the 3 cases (1-3) studied, are given in table III. Column U represents unlabelled

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Table III Percentage distribution of erythroblasts of CDA type I in the different DNA synthesis stages

Table III Percentage															
Case number	Basophilic erythroblasts						Polychromatic erythroblasts				Oxyphilic erythroblasts				
	n	G ₁	G ₂	S	U	H	n	S	U	H	n	S	U	H	
1	413	17.9	10.9	0.5	65.1	5.6	4.1	100	2.0	95.0	37.0	10.7	0.0	100.0	16.8
2	395	14.3	17.9	1.1	64.8	1.9	3.4	170	2.3	97.7	16.5	11.4	0.9	99.1	27.2
3	340	13.5	15.6	0.0	69.7	1.2	1.5	116	4.3	95.7	34.5	11.6	0.0	100.0	17.2
Mean		15.2	14.8	0.5	66.6	2.9	3.0		2.9	97.1	29.3		0.3	99.7	20.4

and octaploid not labelled cells
their DNA content cannot be a

n = number of cells assessed
G₁, G₂, G₃ represent different DNA synthesis stages

S = All ³H TdR labelled cells.

U = Unrecognizable (Unlabelled cells, which on the basis of their DNA content cannot be attributed to one of the resting periods).

H = Labelled and unlabelled hyperdiploid cells.

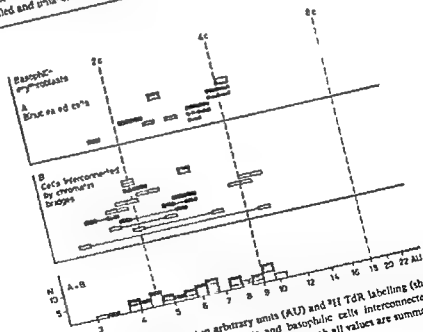


Fig 2 Relative DNA content in arbitrary units (AU) and ³H TdR labelling (shaded areas) in binucleated basophilic erythroblasts and basophilic cells interconnected by chromatin bridges in CDA type I (case 2). In the lower graph all values are summarized

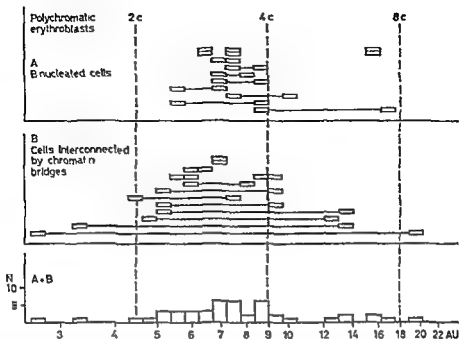


Fig 3 Relative DNA content in arbitrary units (AU) in binucleated polychromatic erythroblasts and polychromatic cells interconnected by chromatin bridges in CDA type I (case 2) In the lower graph all values are summarized ^3H TdR labelling was not observed in these cells

cells, which on the basis of their DNA content cannot be attributed to one of the resting periods. Overall labelling index (S) in the basophilic erythroblasts is between 65 and 70%, and the number of cells in G_1 equals the cell number in G_2 . In the polychromatic and oxyphilic compartment, more than 95% are designated as U cells due to the lack of labelling in these cell types. The percentage of hypertetraploid cells rises with progressing maturation.

Binucleated cells The results of the study of binucleated cells and cells interconnected by thin chromatin bridges in the basophilic and polychromatic compartment are shown in figures 2 and 3. In the lower graphs all nuclei are summarized in a cytogram, in which the same distribution of the DNA content, the different stages of the cell cycle in the basophilic nuclei and the lack of labelling in the polychromatic cells as in the mononucleated cells are demonstrated. The upper graph in figure 2 shows that in the basophilic cells only occasionally both nuclei have a different DNA content. In the polychromatic cells, however, (fig 3, upper graph) the DNA content

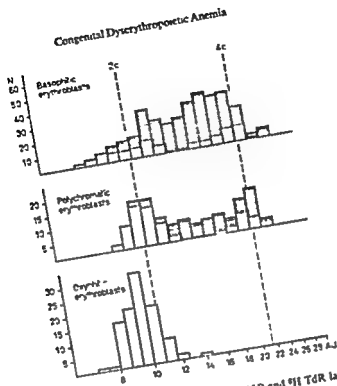


Fig 4 Relative DNA content in arbitrary units (AU) and ^3H TdR labelling (shaded areas) in mononucleated erythroblasts in CDA type II (case 5)

varies considerably and shows diploid and haploid values in the one nucleus and hypertetraploid values in the other one

DNA content and DNA synthesis stages in CDA type II Mononucleated cells The results of the study of one case of CDA type II (case 5) are given in figure 4 The distribution of the DNA content is similar to normal cases as shown by the observation of 2c-4c values in the basophilic and polychromatic cells and diploid values in the oxyphilic cells In the proliferating cell compartments, all stages of the cell cycle as G_1 , S and G_2 are present

The data related to the different stages of the cell cycle in cases 4-6 are given in table IV The overall labelling index (S) ranges from 68-78% G_1 was found to be slightly greater than G_2 within the basophilic cells In the polychromatic erythroblasts the mean labelling index was lower, ranging from 39 to 49%, and the number of G_1 cells was strongly increased ranging from 38 to 46%

Table IV Percentage distribution of erythroblasts of CDA type II in the different stages of the cell cycle

Case number	Basophilic erythroblasts					Polychromatic erythroblasts					Oxyphilic erythroblasts				
	n	G ₁	S	G ₂	U	n	G ₁	S	G ₂	U	n	G ₁	S	G ₂	U
	327	19.9	68.2	11.0	0.9	121	38.0	48.8	12.4	0.8	110	100.0	0.0	0.0	0.0
	324	15.4	71.6	10.2	2.8	116	40.5	46.5	12.1	0.9	101	100.0	0.0	0.0	0.0
	328	16.7	78.5	4.2	0.6	119	46.2	38.7	11.8	3.3	115	100.0	0.0	0.0	0.0
mean		17.3	72.8	8.5	1.4		41.6	44.6	12.1	1.7		100.0	0.0	0.0	0.0
mean normal values (4 cases)		20.8	63.5	15.1	0.6		18.7	65.3	15.3	0.7		96.0	4.0	0.0	0.0

For explanation see table III

Binucleated cells The results of the study in case 5 are given in figure 5. As seen in the lower graphs, the different maturation stages of the nucleated red cells show the same DNA synthesis periods as found in the mononucleated cells. In the polychromatic erythroblasts, the G₁/S ratio is still more shifted in favour of G₁ than in the mononucleated cells. With a few exceptions, DNA content and labelling of the nuclear pairs are concordant.

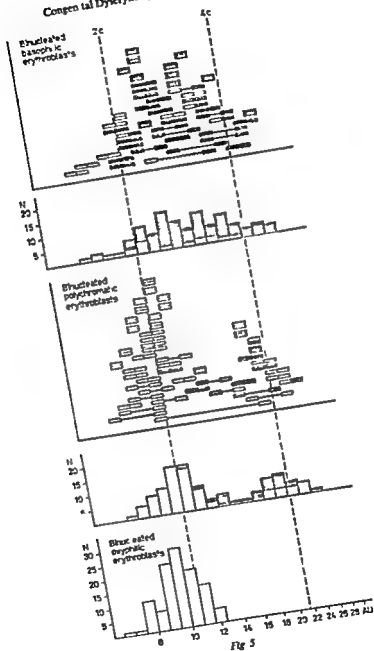
Discussion

The erythropoiesis of 3 nonrelated patients with CDA type I and 3 nonrelated patients with CDA type II has been investigated by combined Feulgen-cytophotometry and ³H-TdR-autoradiography. The results have been identical in each group, but different between the 2 groups, supporting the assumption that type I and type II are different disease entities.

In CDA type I, a complete cell cycle was demonstrated in the basophilic compartment only. The distribution of the different phases of the cell cycle was the same as in normal erythropoiesis, as established by COOPER and WICKRAMASINGHE [1] and by QUEISSER *et al* [11]. Therefore the proliferation in the basophilic compartment appears to be normal, corresponding to the lack of structural and ultrastructural abnormalities [8]. The existence of some

Fig. 5 Relative DNA content in arbitrary units (AU) and ³H TdR labelling (shaded areas) in binucleated erythroblasts in CDA type II (case 5). In the lower graphs all values from 1 cell type are summarized.

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hypertetraploid cells and binucleated cells with different DNA values of the 2 nuclei suggests a disturbance of mitosis in a few cells, which occurs prior to the changes in interphase observed in the more mature erythroblasts

In striking contrast to normal erythropoiesis, the majority of cells containing visible amounts of hemoglobin were unlabelled, and most of them showed a pretetraploid DNA content (fig. 1). This observation indicates an arrest of DNA synthesis in late S-phase. Such an arrest in S-phase has also been described in megaloblastic anemia due to vitamin B₁₂ deficiency [10, 13, 15]. However, in pernicious anemia, DNA synthesis arrest is not restricted to polychromatic cells and the decrease of the labelling index within the polychromatic compartment is by far not as striking as in CDA type I. In addition to the abnormalities in interphase, hypertetraploid mononuclear cells as well as binucleated cells and cell pairs with unequally distributed DNA content were seen in the polychromatic and oxyphilic stage. As stated above, these findings are interpreted as results of disturbance of mitosis.

Electron microscopic studies in CDA type I gave evidence of a defect of the nuclei or nuclear membranes, the erythroblasts showing widening of the membrane pores, condensation and vacuolisation of nuclear chromatin and entrance of cytoplasmic material into the nucleus [8, 9]. On the basis of the data presented, two pathophysiological mechanisms seem to be responsible for the morphological abnormalities and the ineffective erythropoiesis present. Lack of mitosis after normal DNA synthesis, incomplete or abnormal mitosis leads to hyperploid nuclei, nuclear pairs interconnected by chromatin bridges and nuclei with unequally distributed DNA content. This disturbance of mitosis is manifested already in basophilic stages. With increase in hemoglobin concentration, DNA synthesis is arrested, preventing further proliferation. The fact that this interphase abnormality is not manifested in basophilic cells, favors the hypothesis, that hemoglobin intruding into the nucleus interacts with nuclear chromatin and prevents normal DNA synthesis in polychromatic erythroblasts. The arrest of proliferation in the polychromatic compartment is responsible for the failure of production of normal oxyphilic normoblasts which are virtually absent. In cells escaping intramedullary destruction, cytoplasmic maturation may progress without further division.

In CDA type II, no arrest of DNA synthesis during S-phase was observed. The main pathological finding is an increased number of diploid unlabelled polychromatic cells (fig. 4), which was even more pronounced in binucleated erythroblasts (fig. 5). These polychromatic normoblasts showed a small round

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nucleus with structureless chromatin. This observation suggests that DNA synthesis is repressed at a lower hemoglobin concentration than normal [14] however quantitative hemoglobin measurements of these nonproliferating polychromatic normoblasts have not been made. No hypertetraploid cells were present, and the nuclei in binucleated cells had normal equally distributed DNA contents. They are probably the result of normal karyokinesis not followed by cytoplasmic division. It may be speculated, that this is related to the membrane defect present in the red cells of CDA type II as manifested by positive acid serum tests and agglutination by anti i [3, 4].

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Decreased Hemoglobin A₂ Concentration in Iron Deficiency Anemia

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Abstract A comparison of the Hb A₂ concentration in 100 patients with iron deficiency anemia and 100 healthy adults shows a significant decrease in cases with iron deficiency ($p < 0.05$). The mean value was 1.8% for the iron deficient patients and 2.3% for controls. The 2s range was found to be 1.1-2.4% and 1.7-2.9% respectively. No correlation between the amount of Hb A₂ decrease and the degree of anemia and hypochromia could be demonstrated.

Key Words

Hemoglobin A₂
Iron deficiency anemia
Hemoglobin synthesis

The concentration of Hb A₂ in human adults is genetically determined and amounts to 1.7-2.9% of the total hemoglobin. In 1964, CHERNOFF [4] reported several cases of iron deficiency with subnormal concentrations of Hb A₂. Similar cases have since been described by HORTON and HUTSMAN [6] and JONXIS and HUTSMAN [7]. There is therefore evidence that Hb A₂ synthesis is to some extent more inhibited by iron deficiency than Hb A synthesis. However, up to the present time no large scale study has been published. In this paper the Hb A₂ concentrations of 100 patients with iron deficiency anemia are compared with the normal values found in 100 healthy control persons.

Material and Methods

In a cooperative study comprising the departments of internal medicine of 21 Swiss hospitals and 3 general practitioners, 100 cases of iron deficiency anemia have been examined. In all these patients there was a hypochromic, microcytic anemia with MCV below 80 µg and serum iron concentration less than 60 µg%. The 100 healthy control persons have been examined in a survey study for thalassemia. They belong to families in which a thalassemia could be ruled out.

In all iron deficiency and control cases the Hb A₂ concentration was determined in the same laboratory by starch block electrophoresis using Veronal/Veronal Na buffer pH 8.6

The concentration of the eluted Hb A₂ fraction was measured spectrophotometrically in a Beckman DU spectrophotometer at 415 nm

For statistical evaluation arithmetic mean standard deviation and student-dispersion were calculated

Results

The Hb A₂ concentration of 100 patients with iron deficiency anemia is compared with those of 100 healthy adults in figure 1 and the statistical values are represented in table I The arithmetic mean of 1.78% Hb A₂ in iron deficiency and 2.31% Hb A₂ in healthy adults shows a difference which is statistically significant ($p < 0.05$)

A comparison of the Hb A₂ concentration in 50 cases with severe iron deficiency (Hb 2.2–8.5 g%) and in 50 cases with moderate iron deficiency (Hb 8.6–12.2 g%) reveals no difference (fig 2) The arithmetic mean amounts to 1.79% Hb A₂ in the group with severe anemia and to 1.77% Hb A₂ in patients with slight anemia Even the extreme values of 1.1–2.6% Hb A₂ found in cases with marked anemia and 0.9–2.6% Hb A₂ in cases with moderate anemia (fig 2), as well as the standard deviation of 0.353 and 0.309 respectively, show no real difference In figure 3, 50 cases with more

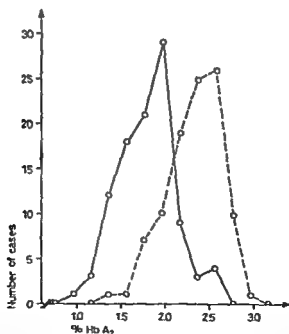


Fig 1 Hb A₂ concentration in 100 adult patients with iron deficiency and 100 adult healthy control persons — = Cases with iron deficiency, - - - = control persons.

marked red cell hypochromia (MCH 9.53–22.2 pg) are compared with 50 cases with only slight hypochromia (MCH 22.3–27.0 pg). Here again, no difference in the Hb A₂ concentration can be found. Arithmetic mean, extreme values and standard deviation are almost identical. The arithmetic mean of the Hb A₂ concentration in the group with more severe hypochromia is 1.79% compared with 1.76% in cases with slight hypochromia. The extreme values are 1.1–2.6% and 0.9–2.6%, the standard deviation 0.359 and 0.302 respectively.

Discussion

Hb A₂ concentration in normal human adults shows nearly a Gaussian curve of distribution. The starch block electrophoresis is a quantitative

Table 1 Hb A₂ in percent of the total hemoglobin in 100 adult patients with iron deficiency anemia and 100 healthy adult controls

	Arithmetic mean %, Hb A ₂	Standard deviation, %, Hb A ₂	2s range, %, Hb A ₂
Iron deficiency	1.78	0.33	1.1–2.4
Healthy adults	2.31	0.23	1.7–2.9

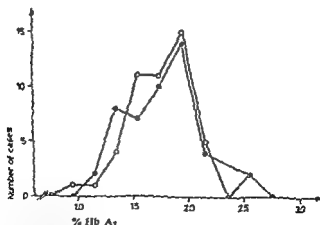


Fig. 2 Hb A₂ concentration and degree of anemia in iron deficiency: ● = Hb 2.1–8.5 g%, ○ = Hb 8.6–12.2 g%.

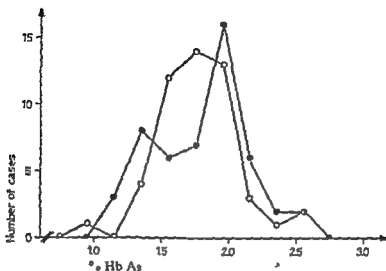


Fig 3 Hb A₂ concentration and degree of red cell hypochromia in iron deficiency
 ● = MCH 9.53-22.2 pg, ○ = MCH 22.3-27.0 pg

method for Hb A₂ determination with an accuracy not surpassed by any other technique. The normal 2s range in adult blood found in our laboratory is 1.7-2.9% Hb A₂ and agrees quite well with the values found by CHERNOFF [4] and JONXIS and HUISMAN [7] using chromatographic methods. CHERNOFF found a normal range of 2.1-2.9% Hb A₂, while JONXIS and HUISMAN reported values of 1.5-3.0% Hb A₂.

Decreased Hb A₂ concentrations have been described in a number of acquired red cell disorders. In addition to the above mentioned cases of iron deficiency, FLATZ *et al* [5] observed low Hb A₂ concentrations in infections with *Ankylostoma duodenale*, which may be explained as a result of iron deficiency. REED and MOLLIN [9] reported subnormal concentrations of Hb A₂ in 13 patients with sideroblastic anemia. In 4 of these cases, the Hb A₂ concentration was less than 1%. It follows therefore, that a Hb A₂ decrease can be found in different disorders of iron metabolism. An increased concentration of Hb A₂ in cases with acquired red cell disorders has been described as well. ARENDS [1, 2] published 15 cases of malaria with 3.0-3.8% Hb A₂. VAN ROS *et al* [10] found a slight Hb A₂ increase in certain cases of schistosomiasis before, as well as after treatment. A slightly increased Hb A₂ concentration in untreated pernicious anemia was first described by JOSEPHSON *et al* [8]. JONXIS and HUISMAN [7] reported 3 cases of megaloblastic anemia with 3.0-3.5% Hb A₂. REED and MOLLIN [9] found a slight increase of Hb A₂ in 70% of patients with megaloblastic anemia and finally BRIDGES

et al [3] observed a temporary Hb A₂ increase in 3 patients 10-20 days after homograft of fetal hematopoietic tissue

The results of this survey confirm, as far as we are aware, for the first time at a statistically significant level, that iron deficiency reduces the Hb A₂ concentration to a greater extent than the Hb A concentration. However, a quantitative correlation between the degree of anemia or hypochromia and a corresponding decrease in the Hb A₂ concentration cannot be demonstrated. The mechanism responsible for the preferential decrease of Hb A₂ synthesis in iron deficiency is not known. Hb A₂ is the latest hemoglobin phylogenetically appearing first in primates, and the latest hemoglobin in human ontogenesis and is produced only in small quantities during lifetime. The synthesis of this hemoglobin component is mostly affected by iron deficiency.

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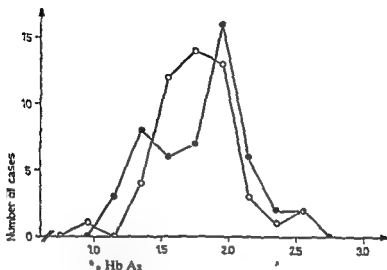


Fig 3 Hb A₂ concentration and degree of red cell hypochromia in iron deficiency
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RNA and Protein Synthesis in Proliferating and Non-Proliferating Blast Cells of Human Acute Leukaemia

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Abstract Cell size can be used to distinguish between the proliferative and the non proliferative or resting cells in human acute leukaemia. The large, proliferative blast cells not only have a higher rate of RNA synthesis, but also synthesise more labile RNA, compared to the smaller, resting cell. No difference in the life time of templates was found between the two cell types.

Key Words

Actinomycin D
Autoradiography
Cell culture
Cell size and proliferation
Leukaemic cells
RNA synthesis

Recent evidence suggests that the proliferative cell cycle consists of a sequence of biochemical steps, some of which, including the critical decision to initiate the process of DNA replication, involve the synthesis of short-lived messenger RNA [1-4]. The cell population in human acute leukaemia is not a uniformly rapidly proliferating cell population [5-8], but may be regarded as consisting of proliferative and non-proliferative cells. The present work investigates RNA synthesis and degradation and their relationship to protein synthesis in the two cell types.

Material and Methods

Cells for culture were obtained from marrow or blood of patients with acute myeloid leukaemia (6 cases) and acute lymphoblastic leukaemia (6 samples from 5 cases). 8 of these samples (2 myeloid, 4 lymphoblastic) were studied in greater detail. All the samples contained > 85% of blast cells. The culture medium contained TC 199 (Glaxo) 70% and either autologous plasma or AB Rh +ve serum 30%. Labelled compounds were obtained from the Radiochemical Centre, Amersham and their specific activities and concentrations in the cultures were as follows: ^3H thymidine 5 Ci/mm, 1 $\mu\text{Ci/ml}$, uridine-5- ^3H 5 Ci/mm.

2 μ Ci ml. 3 H leucine 0.5 Ci mCi, 10 μ Ci ml Actinomycin D (AMD) was obtained from Merck, Sharpe and Dohme Ltd., and used in a concentration of 4 μ g/ml

Preliminary experiments using nuclease digestion established that most of the label in uridine- 3 H appeared in RNA and less than 3% in DNA. The dosage of AMD used suppressed >95% of the RNA synthesis in these cells

After incubation with radioactive chemicals as detailed below the cultures were sampled and smeared on glass slides. Autoradiographs were then prepared, using Ilford G5 emulsion. In the 3 H-thymidine experiments, the labelling index (% of cells labelled) was determined by counting 2,000 cells from each autoradiograph. In the other experiments, grain counts were made on 50 cells from each autoradiograph. The labelling index was also determined by counting 500 cells and a correction was then made to the mean grain count. Cell size was measured using an eye-piece graticule calibrated on a stage micrometer. The longest and shortest cell diameters were measured and a mean was taken.

Results

In 12 experiments the cultures were incubated with 3 H thymidine for 1 h, the sizes of labelled and unlabelled cells were then compared. In each case the labelled cells were significantly larger than the unlabelled cells (table 1). This suggests that cell size is a useful criterion for separating the proliferative and non-proliferative cell populations.

Table 1 Comparison of sizes of 3 H thymidine labelled and unlabelled cells in 12 leukaemic cells samples. The number of cells scored in each group is 30

Sample number	Labelling index, %	Labelled cells diameter, μ m (mean \pm SD)	Unlabelled cells diameter μ m (mean \pm SD)	Significance of difference
1	4.0	12.51 \pm 0.91	10.02 \pm 1.45	B 0.001
2	13.8	12.76 \pm 0.89	10.29 \pm 1.46	P < 0.001
3	9.5	11.99 \pm 1.21	9.98 \pm 1.12	P < 0.001
4	5.4	12.61 \pm 1.13	10.41 \pm 1.45	P < 0.001
5	18.4	12.80 \pm 1.21	10.31 \pm 1.38	P < 0.001
6	8.2	12.42 \pm 1.02	9.95 \pm 1.24	P < 0.001
7	11.0	12.61 \pm 1.19	9.94 \pm 1.21	P < 0.001
8	14.5	12.21 \pm 1.01	9.82 \pm 1.14	P < 0.001
9	9.6	12.46 \pm 1.05	10.05 \pm 1.60	P < 0.001
10	10.4	12.40 \pm 1.02	10.08 \pm 1.40	P < 0.001
11	15.0	12.00 \pm 1.21	10.12 \pm 1.26	P < 0.001
12	5.6	12.52 \pm 1.00	10.18 \pm 1.34	P < 0.001

RNA and Protein Synthesis in Proliferating and Non-Proliferating Blast Cells of Human Acute Leukaemia

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Abstract Cell size can be used to distinguish between the proliferative and the non-proliferative or resting cells in human acute leukaemia. The large, proliferative blast cells not only have a higher rate of RNA synthesis, but also synthesise more labile RNA, compared to the smaller, resting cell. No difference in the life time of templates was found between the two cell types.

Key Words

Actinomycin D
Autoradiography
Cell culture
Cell size and proliferation
Leukaemic cells
RNA synthesis

Recent evidence suggests that the proliferative cell cycle consists of a sequence of biochemical steps, some of which, including the critical decision to initiate the process of DNA replication, involve the synthesis of short-lived messenger RNA [1-4]. The cell population in human acute leukaemia is not a uniformly rapidly proliferating cell population [5-8], but may be regarded as consisting of proliferative and non-proliferative cells. The present work investigates RNA synthesis and degradation and their relationship to protein synthesis in the two cell types.

Material and Methods

Cells for culture were obtained from marrow or blood of patients with acute myeloid leukaemia (6 cases) and acute lymphoblastic leukaemia (6 samples from 5 cases). 6 of these samples (2 myeloid, 4 lymphoblastic) were studied in greater detail. All the samples contained >85% of blast cells. The culture medium contained TC 199 (Glaxo) 70%, and either autologous plasma or AB Rh + ve serum 30%. Labelled compounds were obtained from the Radiochemical Centre, Amersham and their specific activities and concentrations in the cultures were as follows: ^3H thymidine 5 Ci/mM, 1 $\mu\text{Ci}/\text{ml}$, uridine 5 ^3H 5 Ci/mM,

2 $\mu\text{Ci ml}$, ^3H leucine 0.5 Ci mu, 10 $\mu\text{Ci ml}$ Actinomycin D (AMD) was obtained from Merck, Sharpe and Dohme Ltd., and used in a concentration of 4 $\mu\text{g ml}$.

Preliminary experiments using nuclease digestion established that most of the label in uridine-5- ^3H appeared in RNA and less than 3% in DNA. The dosage of AMD used suppressed > 95% of the RNA synthesis in these cells.

After incubation with radiochemicals as detailed below, the cultures were sampled and smeared on glass slides. Autoradiographs were then prepared using Ilford G5 emulsion. In the ^3H -thymidine experiments, the labelling index (% of cells labelled) was determined by counting 2,000 cells from each autoradiograph. In the other experiments, grain counts were made on 50 cells from each autoradiograph. The labelling index was also determined by counting 500 cells and a correction was then made to the mean grain count. Cell size was measured using an eye-piece graticle calibrated on a stage micrometer. The longest and shortest cell diameters were measured and a mean was taken.

Results

In 12 experiments the cultures were incubated with ^3H -thymidine for 1 h; the sizes of labelled and unlabelled cells were then compared. In each case the labelled cells were significantly larger than the unlabelled cells (table 1). This suggests that cell size is a useful criterion for separating the proliferative and non-proliferative cell populations.

Table 1 Comparison of sizes of ^3H thymidine labelled and unlabelled cells in 12 leukaemic cell samples. The number of cells scored in each group is 30.

Sample number	Labelling index, %	Labelled cells diameter, μm (mean \pm SD)	Unlabelled cells diameter, μm (mean \pm SD)	Significance of difference
1	4.0	12.51 \pm 0.91	10.02 \pm 1.45	$P < 0.001$
2	13.8	12.76 \pm 0.89	10.29 \pm 1.46	$P < 0.001$
3	9.5	11.99 \pm 1.21	9.93 \pm 1.12	$P < 0.001$
4	3.4	12.61 \pm 1.13	10.41 \pm 1.45	$P < 0.001$
5	18.4	12.80 \pm 1.21	10.31 \pm 1.39	$P < 0.001$
6	8.2	12.42 \pm 1.02	9.93 \pm 1.24	$P < 0.001$
7	11.0	12.61 \pm 1.19	9.94 \pm 1.21	$P < 0.001$
8	14.5	12.21 \pm 1.01	9.82 \pm 1.14	$P < 0.001$
9	9.6	12.46 \pm 1.05	10.05 \pm 1.40	$P < 0.001$
10	10.4	12.40 \pm 1.02	10.03 \pm 1.40	$P < 0.001$
11	15.0	12.00 \pm 1.21	10.12 \pm 1.26	$P < 0.001$
12	5.6	12.52 \pm 1.00	10.18 \pm 1.34	$P < 0.001$

Table II Correlation between cell diameter and intensity of ^3H uridine labelling

Sample number	Cell diameter, μm (mean \pm SD)	Grain count (mean \pm SD)	r^1
2	10.30 \pm 1.51	72.82 \pm 44.85	0.58
3	10.92 \pm 1.47	46.18 \pm 38.57	0.75
7	10.27 \pm 1.60	30.10 \pm 27.88	0.75
8	10.89 \pm 1.82	49.38 \pm 37.35	0.74
9	10.22 \pm 1.71	35.42 \pm 31.09	0.82
10	10.68 \pm 1.93	40.26 \pm 34.71	0.83

¹ $P < 0.001$

In the 6 cell samples selected for more detailed study, paired cultures were set up, one incubated with ^3H -thymidine for 1 h and the other with ^3H -uridine for 30 min. In all 6 cases there was a significant correlation between cell size and the intensity of ^3H -uridine labelling (table II). Two typical examples are illustrated in figures 1 and 2, which also show the size-distribution of ^3H -thymidine labelled cells. These results suggest that the larger, proliferative cells are also engaged in an increased rate of RNA synthesis, whereas the smaller resting cells are much less active in the synthesis of RNA.

In the 6 cultures incubated with ^3H -uridine for 30 min, further RNA synthesis was stopped by adding AMD to the cultures, and the decline of radioactivity in the cells was followed for 4 h. This decline was studied separately in the larger proliferative cells ('P' cells, defined as having a mean cell diameter of $> 11 \mu\text{m}$) and the smaller resting cells ('R' cells, defined as having a mean cell diameter of $< 10 \mu\text{m}$). In the corresponding cultures incubated with ^3H -thymidine, determination of the labelling index separately for 'P' and 'R' cells (table III) shows that the proportion of 'P' cells synthesising DNA was much higher than the proportion in the cell population as a whole, whereas in 5 of the 6 cases no DNA synthesising cells were included in the 'R' group and in the remaining case only a very small proportion of such cells were included. These results suggest that a good degree of separation between the proliferative and resting cell populations was achieved.

The decline in RNA radioactivity following AMD treatment in the two cell types is shown in figure 3. The rate of degradation of rapidly-labelled RNA appears much greater in the larger proliferative cells.

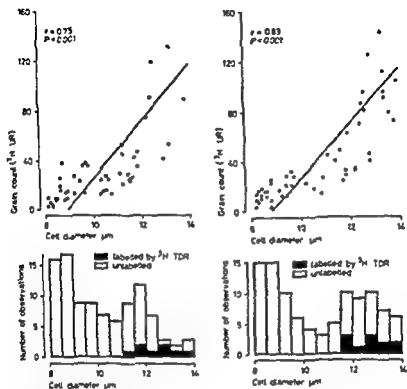


Fig 1 and 2 Relationship between cell size and RNA synthesis (top graph) and between cell size and DNA synthesis (bottom graph) in two leukaemic cell samples studied by the paired culture technique

The effect of the inhibition of RNA synthesis by AMD on the synthesis of proteins was also studied in 2 of the 6 cell samples. Cultures were prepared and incubated with ^3H leucine for 1 h. AMD was then added and the cultures were sampled at intervals during the next 4 h. Results for P and II cells are shown in figure 4. Differences in intensity of labelling between the cell types were not statistically significant. In both cell types protein synthesis continued for about 90 min after the addition of AMD but after this time very little further synthesis occurred. In a control culture not treated with AMD incorporation of ^3H Leu continued at a near uniform rate throughout a period of 4 h. These experiments thus revealed no

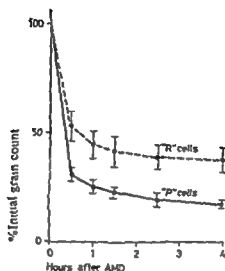


Fig 3 RNA degradation in proliferative (P) and resting (R') cell populations

Table III ^3H thymidine labelling index (LI) in proliferative (P) and resting (R') cells

Sample number	LI in whole sample, %	P cells		R' cells	
		% of whole sample	LI, %	% of whole sample	LI, %
2	13.8	30	35.4	56	3.1
3	9.5	48	22.5	31	0
7	11.0	36	29.6	51	0
8	14.5	46	29.4	37	0
9	9.6	32	25.0	56	0
10	10.4	47	21.2	46	0

differences in the life-time of templates for protein synthesis in the two cell types

Discussion

The finding that cell size can be used to differentiate between proliferative and non-proliferative or resting cells in human acute leukaemia is in agreement with the work of MAUER and FISHER [9] and GAVOSTO *et al* [10, 11]. That the larger leukaemic cells are more active in RNA synthesis is also supported by the report of FOADI *et al* [12].

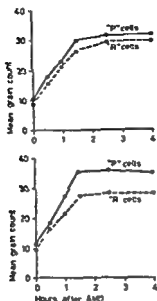


Fig. 4 Effect of AMD on protein synthesis in proliferative (P⁺) and resting (R⁺) cells (2 studies)

The conclusion of chief interest in the present work is that the proliferative leukaemic cells, as well as having a high rate of RNA synthesis, also synthesise more labile RNA. This is consistent with the hypothesis that cell proliferation is a special example of the expression of genetic information, i.e., that information for cell proliferation is encoded in nucleotide sequences of DNA. When these genes for cell proliferation, which BULLOGHI [13] has called the 'mitosis operon', are activated, for example via a feedback mechanism in terms of the cell population size, the synthesis of messenger RNA occurs, which in turn directs the synthesis of enzymes essential for proliferation, e.g. thymidine kinase. Such messenger RNA may be expected to have the properties of being rapidly labelled and rapidly degraded. However, it must also be borne in mind that not all rapidly-labelled RNA can be assumed to have messenger function [14].

The failure to demonstrate long-lived templates for protein synthesis is not unexpected. Such long-lived messenger RNA are generally found in cells highly specialised for tissue function, e.g., liver cells [15] and reticulocytes [16] and there is no evidence that the non-proliferative leukaemic cell

is a biochemically specialised cell and no tissue function can be postulated for it

Acknowledgements I am grateful to Prof F G J HAYHOE for advice and to Mr R J FLEMANS and Miss CAROL SMITH for excellent technical assistance. This work was supported by an Elmore Research Studentship

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Lymphoid Myelofibrosis

About 10 Further Observations

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Abstract Lymphoid myelofibrosis represents an anatomoclinical syndrome of constant symptomatology characterized predominantly by splenomegaly, few lymphadenopathies, pancytopenia with blood lymphocytosis, and a hyperplastic myelogram comprising lymphocytes and histiocytes. Histological study of bone marrow biopsy shows association of lymphoid proliferation as well as network cell proliferation giving rise to a dense lattice of reticulum and collagen fibers, without osteosclerosis. The cytology of the syndrome and its relationship to lymphoid leukemia, Waldenström's disease and those syndromes termed reticuloendotheliosis or malignant histiocytosis is discussed. It is not easy to evaluate the efficiency of therapeutic measures since myelofibrosis precludes that kind of chemotherapy as is currently used in lymphoproliferative syndromes. Therefore prognosis is uncertain although clinical evolution is slow.

Key Words:
Bone marrow biopsy
Lymphoid myelofibrosis
Myelofibrosis
Pancytopenia
Reticulosis

In 2 previous publications [10, 11] we singled out among various lymphoproliferative syndromes one clinical and hematological picture characterized by inconstant lymph node enlargement, fairly constant spleen enlargement, pancytopenia with relative lymphocytosis, and constant hypoplasia of the myelogram that contained over 50% lymphocytes. The converging point of these findings is systemic myelofibrosis, easily demonstrated by histological studies of the bone marrow. In such myelofibrosis normal medullary tissue has disappeared, replaced by lymphoreticular proliferation appearing in an even pattern through the network of fibrosis. In this syndrome several diseases described in the literature under such various titles as aleukemic lymphocytopenic leukemia, spleen sarcomatosis, medullar histiocytic reticulosis, malignant histiocytosis, etc., appear to center round myelofibrosis.

Table I Peripheral blood values

	Case number									
	1	2	3	4	5	6	7	8	9	10
Erythrocytes $\times 10^6$	2.1	3.6	3.2	2.3	3.2	3.9	2.5	2.8	2.3	3.2
Hb, g%	6.5	9	9	7.2	9.8	10.7	8	8.8	7.7	10.2
Reticulocytes, %	1.8	1.2	0.5	2	0.1	0.5	1.2	0.8	3.2	2.4
Leucocytes	1 000	2 500	1 400	1 500	1 100	1 300	2 200	2 300	3 200	2 400
Neutrophils	10	40	3	22	23	37	13	57	33	16
Eosinophils	8	2		2						
Basophils	4			1						
Large Lymphocytes	74	23	81	69	60	11	45	30	50	49
Small Lymphocytes	4	4			16	51	34	5	12	22
Lymphoblasts	2	29	13	3	1		4	5	3	11
Monocytes			3	3		1	4	3	2	2
Plasmacytes		2								
Thrombocytes	110 000	80 000	5 000	100 000	50 000	70 000	90 000	80 000	120 000	25 000

In several subsequent publications attempts have been made to mark out its limits and to emphasize the relationship between myelofibrosis and lymphoid proliferation [1, 2, 4, 5, 7-19, 21-24]. We would like to resume the discussion, in connection with 10 further observations, of which the hematological characteristics are summarized in tables I and II.

Clinical and Hematological Synthesis

The 10 observations, together with others previously published, corroborate the clinical, hematological, and histological features of lymphoid myelofibrosis. The disease is observed most often in males, especially after 50 years of age. Lymph node enlargement is not always seen. On the other hand, the spleen is usually enlarged although not often bulky. Deep-seated nodes do not seem to be involved. In no case were mediastinal adenopathies observed. Lymphography which was carried out in 2 cases, was normal.

Pancytopenia is a most striking and baffling feature in this syndrome, it may lead to an inaccurate diagnosis of bone marrow failure. Anemia, often severe, occurs early and shows no signs of regeneration. Leukopenia and granulopenia are customary findings, yet we observed one case with

Table II Bone marrow differentiation

	Case number									
	1	2	3	4	5	6	7	8	9	10
Neutrophils	6	18	4	2	7	5	11	3	4	12
Eosinophils	2	1	1			1		1		
Basophils	1				2					
Metamyelocytes	1	5	11	2	3	12	8	3	3	5
Myelocytes	5	6	12	2	5	5	7	2		3
Promyelocytes	2	7	4	2		2	1			
Myeloblasts					7					1
Lymphocytes	44	27	40		54	18	24	32	50	57
Lymphoblasts	39	35	11	44		20	5	47	43	8
Monocytes			4					4		2
Plasmacytes		1	1	3	5	1	1	2	2	2
Naked nuclei			12	45	17	36	42	8		10
Erythroblasts	33	23	12	49	5	36	26	4	2	28

marked initial leukocytosis and WARTZ *et al* [24] have also reported 2 such cases with a high rate of leukocytosis. Such leukocytosis eventually disappears during evolution. Blood lymphocytosis involved mainly the large and the small lymphocytes with few lymphoblasts. The platelet count was always decreased.

The myelogram of aspirated material was always hypoplastic, a fact which may increase the hazard of its being mistaken for medullary failure. There was a preponderance of lymphoid and histiocytic cells. The types of cells will be further discussed below. Myelofibrosis, constantly revealed by bone marrow biopsy, accounts for the apparent hypoplasia of the myelogram. The reticulin network, on silver staining, appears much developed, forming a tight lattice which surrounds almost every cell. Its fibers are thickened and largely branched. The lattice is lined with a less regular, looser, lattice of collagen, visualized on trichrome staining (fig 1 and 2). In the mesh, normal myeloid cells have almost totally disappeared, replaced by populations of lymphocytes, histiocytes and reticular cells, loosely arranged between the meshwork of myelofibrosis. There is no osteosclerosis (fig 3).

The spleen which was examined in 2 cases, is the seat of lymphoid and reticular proliferation, predominantly immature forms. The reticulinic texture of the red pulp is increased. Liver biopsy revealed hyperplasia of the Kupffer cells and large numbers of lymphocytes in the sinuses in 2 cases.

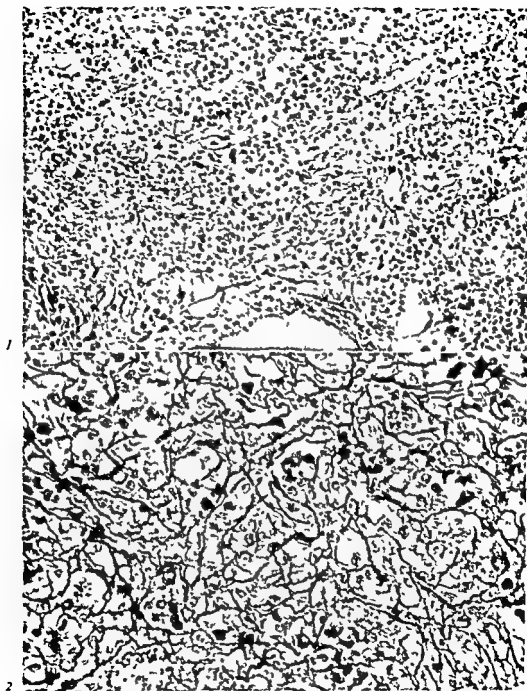


Fig 1 Bone marrow biopsy trichrome coloration. Medullary tissue is replaced by dense felt like reticulin and collagen fibres. Loosely scattered lymphoid cells are seen in the network. Note active dilatation of medullary sinuses ($\times 180$)

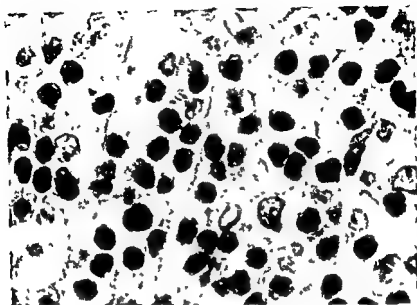


Fig 3 Bone marrow biopsy, hematin-eosin. Lymphocytes, lymphoblasts, and a few reticular cells are seen in thickened medullary lattice ($\times 1,200$)

The lymph nodes, studied in 2 previous cases, show lymphatic and lymphoblastic proliferation, with increased density of the reticulinic texture.

Thus, all hemopoietic organs exhibit the same bipolar proliferation involving both the lymphoid line and the cells generative of the stromal fibers of the organs.

There is an elevation in immunoglobulins in most cases, but it is an uneven one, without any evidence of monoclonal production (fig 4). The ESR is increased in almost all cases.

Diagnostic Discussion

The cytologic problem. While myelofibrosis constitutes the most striking and constant characteristic, the associated cell proliferation may often give rise to controversy, owing to its polymorphism.

Fig 2 Bone marrow biopsy, Gomori. Extremely dense reticulin network, almost every cell is surrounded by the mesh ($\times 480$)

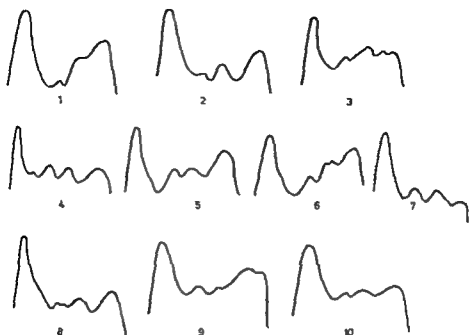


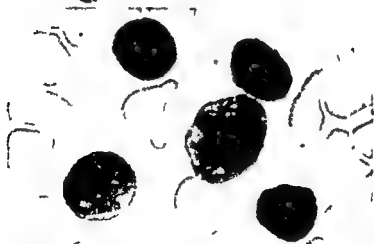
Fig 4 Diagram of serum electrophoresis in cases 1-10

In the blood the lymphocytes are moderately increased in absolute number. An actual increase was found in one case only (case 1). Large lymphocytes are clearly predominating, in 7 of 10 cases they constitute more than 50% of the cells seen on smears. The small lymphocytes are relatively less increased. The presence of lymphoblasts in the blood is constant. The rather large, rounded, nucleus contains 1 or 2 nucleoles (fig 5). The high percentage of these, led to an inaccurate diagnosis of acute lymphoblastic leukemia in one case. As opposed to such lymphocytosis, the monocytes are normal or even reduced in number, which rules out the hypothesis of primitive reticular or endothelial proliferation. The lymphoid nature of cells in the blood is further confirmed by electronmicroscopic studies which show the nuclear structure to be of a lymphocytic type in 2 cases. Also, the macrophagic test of carbon particles by those cells shows but very weak trapping, thus excluding their being of a monocytic nature. These lymphocytes are PAS negative. In culture with PHA they transform normally into blast cells on the third day.

On bone marrow smears – always poor, owing to myelofibrosis – a similar lymphocytic and lymphoblastic proliferation is found. A fairly important number of mesenchymal cells is also seen, indicating the involve-



5



6

Fig 5 Peripheral blood. Clusters of lymphocytes and lymphoblasts ($\times 1,200$)

Fig 6 Bone marrow. Lymphocytes, lymphoblast, reticular cell ($\times 1,200$)

ment of the medullary network in the tissue damage (fig 6). Such cells are polymorphous. They sometimes resemble reticular cells with a finely beaded chromatin; more often they are histiocytes with a smoother chromatin, a round, often nucleated nucleus and a narrow, pale blue-staining cytoplasm.

sometimes fibroblastic or endothelial differentiation is visualized on smears. There are always large numbers of bare nuclei indicating that such tissue cells have been torn from the medullary network. Thus, such cellular proliferation as a whole suggests a dual polarity, lymphoid and reticulohistiocytic. This is explained by a study of the framework of the hemopoietic tissue, the most evident and accessible example of which is provided by bone marrow biopsy.

The myelofibrosis problem The involvement of the reticulin and the collagen network in tissue damage is the most significant feature of lymphoid myelofibrosis. But only by histological examination of the bone marrow will this symptom be demonstrated, since myelogram entirely fails to do so.

Histological examination distinguishes between 2 categories of cells in such dense network: on the one hand, reticular and histiocytic cells that contribute in the formation of the lattice, on the other hand lymphoblasts and lymphocytes, arranged in clumps in its mesh. A similar type of damage is also seen in spleen and lymph nodes [11].

Thus, the parallel proliferation of the network and its lymphoid cell content accounts for the cellular polymorphism in the disease and the difficulties in an accurate nosologic classification, depending upon whether lymphoid- or reticulo-histiocytic cells predominate in the samples. Histological study of the bone marrow shows both types to be present in constant association.

The nosologic problem The histologic and cytologic characteristics as a whole differentiate lymphoid myelofibrosis from other lymphoproliferative syndromes. The disease is entirely distinct from chronic lymphocytic leukemia, which is never pancytopenic, where no lymphoblasts are seen, and where lymphocytes constantly exhibit immunologic deficiency. We never in our experience found an increased density of the bone marrow network in lymphocytic leukemia and this is indeed a characteristic shared in common by all types of leukemias, such diseases involving solely the free hemopoietic cells. Lymphoid myelofibrosis is also distinct from Waldenström's disease, of which it lacks the secretor character, despite an often elevated immunoglobulin level. However, both share a few common characteristics since, in Waldenström's disease, the cytology is often polymorphic and the reticulin network of the bone marrow is often denser. Thus, Waldenström's disease also involves – though more partially – the cells of the hemopoietic framework.

Most likely, lymphoid myelofibrosis groups together several data previously described under the appellations of leukemic reticuloendotheliosis [3].

histiocytic leukemia [6] or malignant histiocytosis [20] where similar features, clinical as well as hematological, are found: frequent splenomegaly, blood pancytopenia, hypoplasia of the myelogram, presence of lymphoid and histiocytic cells in the blood and bone marrow. In fact, in such observations histological studies of the bone marrow are lacking. These would most likely show the participation of the network, which goes on a par with histiocytic proliferation.

Some would insist that circulating cells are of an entirely histiocytic nature in the syndrome [2]. Such trend seems an exaggeration, considering the cellular polymorphism observed, and keeping in mind the nonparticipation of the monocytes which represent the circulating form of reticulo-histiocytic cells. The histiocyte, by definition, is not a circulating cell, and its presence here brings evidence for the proliferation of the framework in a syndrome which, in other respects, is essentially lymphoid.

Practical Consequences

The understanding of lymphoid myelofibrosis is not of purely theoretical interest. It makes it possible to gather together under a single pattern of tissue damage, several blood disorders frequently encountered, but as yet too ill understood for a precise line of conduct to be adopted in instituting therapy or establishing prognosis. The finding of myelofibrosis should in effect inspire the utmost caution in therapeutic indications. Chemotherapy, currently used in lymphoproliferative syndromes, entails a possibility of prompt aggravation of cytopenia, and earlier occurrence of blood aplasia. Moreover the evolution of myelofibrosis is very slow and the disease may, for protracted periods, require no treatment. However, the frequent occurrence of aplastic complications makes its prognosis rather severe despite the usual slowness of evolution.

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Significance of Abnormal LDH Isoenzyme Pattern in Human Platelets

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Medical Department (Chefs: R. FRIBERG and J. GORWEN) Coagulation Laboratory
Sundby Hospital Copenhagen

Abstract The distribution of lactate dehydrogenase (LDH) isoenzymes in platelets from 41 normal persons and 59 patients was studied by disc polyacrylamide electrophoresis. The results were analysed statistically. Compared with the normal controls, a group of bleeding/surgical patients, expected to exhibit a predominance of young platelets, showed a significant decrease in the ratio between H and M monomers (decrease in T value). With a group of myelo- and lymphoproliferative disorders, normal T values were found in essential thrombocythaemia, chronic myeloid leukaemia, blastoid leukaemia and chronic lymphatic leukaemia. In myelofibrosis T values were significantly decreased while in polycythaemia vera they were increased.

Key Words

LDH in thrombocytes
Polyacrylamide electrophoresis
Thrombocyte enzymes

Normal human platelets exhibit a special lactate dehydrogenase (LDH) isoenzyme pattern [10, 13, 23, 24, 30]. A decrease in the ratio between H and M monomers has been demonstrated especially in patients suffering from metastatic cancer or acute phlebothrombosis [13]. The underlying mechanism might be a shift in mean platelet age and/or intrinsic defects of platelets and/or platelet precursors.

By means of disc polyacrylamide electrophoresis, the present study aimed at evaluating the LDH isoenzyme pattern in platelets from a group of bleeding/surgical patients, expected to exhibit a predominance of young platelets [1, 5, 9, 20], and from patients suffering from myelo- and lymphoproliferative disorders, which are often associated with biochemical abnormalities of the platelets [2, 8, 17, 28].

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Table 1 Average T values, Standard deviations and p-values in the total material

Diagnosis	Cases	T values	SD	p
Normal controls	41	0.19	0.04	
Surgical controls	7	0.19	0.03	0.3
Bleeding surgical group	21	0.15	0.03	= 0.01
Stored blood	6	0.27	0.04	= 0.001
Myelo- and lymphoproliferative disorders	25			
Essential thrombocythaemia	3	0.16	0.06	> 0.2
Chronic myeloid leukaemia	5	0.17	0.02	> 0.2
Myelofibrosis	6	0.14	0.02	= 0.01
Polycythaemia vera	2	0.25	0.03	= 0.02
Blastic leukaemia	4	0.20	0.01	> 0.2
Chronic lymphatic leukaemia	5	0.17	0.06	> 0.2
Total	100			

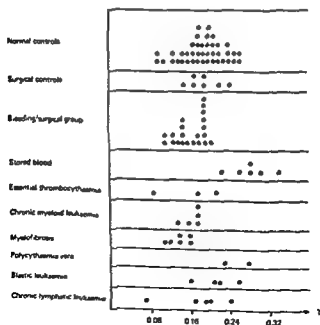


Fig. 1 T values in the total material. Abscissa T = log. H/V

Compared to the T-values of the normal controls, those of the surgical controls did not show significant differences ($p > 0.3$), whereas the T-values in the bleeding/surgical group were significantly lower ($p = 0.01$). High T-values were found in stored platelets ($p = 0.001$).

Within the group of myelo- and lymphoproliferative disorders, normal T-values were found in essential thrombocythaemia, chronic myeloid leukaemia, blastic leukaemia, and chronic lymphatic leukaemia. In myelofibrosis T-values were significantly decreased ($p = 0.01$), while in polycythaemia vera they were increased ($p = 0.02$).

Discussion

The investigations were done to clarify our previous observations of abnormal LDH isoenzyme patterns in human platelets related to metastatic cancer and acute phlebothrombosis [13].

The factors influencing the ratio between H and M monomers in platelets are unknown. Platelet T-values might merely reflect the cytoplasmic LDH isoenzyme distribution of the mature granular megakaryocyte. However, the T-values of platelet precursors are unexplored.

Another mechanism might be operative, i.e., a change in T-values during the lifespan of the platelets. Transition from cathodal to anodal forms of isoenzymes in the bovine lens is associated with cellular ageing [27]. Age-dependent T-values have been demonstrated in erythrocytes, probably correlated to faster inactivation of M monomers during ageing *in vivo* [25]. If the same applies to the platelets, T-values might be decreased in young, increased in old platelets.

Protein synthesis in the platelets seems limited [11, 12] and decreases during their lifespan [26]. H and M monomer synthesis, based on the existence of stable messenger RNA in the platelets [31], can hardly be believed to play any significant role in the shifts in T-value during the lifespan of the platelets. However, no data concerning this problem are available. Furthermore, T expresses a relationship between enzymatic activities, a simple correlation between activity and amount of H and M does not necessarily exist. Finally, it cannot be excluded that cellular environments are able to exert an effect on the isoenzyme pattern by influencing the stability of the monomers in the tetrameric state [3].

The present study demonstrated abnormally low T-values in a bleeding/surgical group, known to exhibit an excess of young platelets. Low T-values were previously found in metastatic cancer and in acute phlebothrombosis

[13], disorders often combined with non-specific thrombocytosis [18], probably due to young platelets. This supports the view that T-values might be related to mean platelet age.

The high T-values found in stored blood agree with the well-substantiated instability of the M monomer to cold *in vitro* [16]. The low T-values found in the bleeding/surgical group were not due to blood transfusions.

Most previous authors have reported normal or slightly shortened platelet survival in myeloproliferative disorders [21]. As a shortened platelet survival has been found in cross transfusion studies, it has been suggested that platelets from such patients have intrinsic defects [17]. Abnormal platelet alkaline phosphatase activity [2] and zymogram [28] have been reported in myeloproliferative conditions. The low T-values found in myelofibrosis might be correlated to a shortened survival of the platelets, but the high parameters in polycythaemia vera can hardly be associated with a shift in platelet age, as no well documented prolonged survival has been described in such cases [8]. The heterogeneity of T-values in myelo- and lymphoproliferative disorders is so far unexplained. The sub-band pattern of the LDH isoenzymes was previously found to be normal in myeloproliferative disorders [14].

The present results are suggestive of a correlation between T-values and platelet age, although not all data are compatible with the theory. As young and old platelets exhibit essential morphological [11, 12], biochemical [6, 11], functional [12, 19], and kinetic [11] differences, studies of T-values in extreme platelet cohort populations would be of interest. Such investigations are in progress.

Acknowledgement This investigation was aided by grants from "Statens Lægevidenskabelige Forskningsråd", "Den lægevidenskabelige forskningsfond for Storkøbenhavn, Færøerne og Grønland", and "Statens almindelige Videnskabsfond". The author is greatly indebted to Mrs. JANE PEDERSEN for technical assistance and to Dr. TET TILR, for statistical assistance.

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Carrier Proteins of Folic Acid Activity in Human Serum

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Abstract The natural folic acid activity (FAA) in the serum, on Sephadex® G 200 gel filtration, is divided into 3 fractions: the first in the band of large molecular proteins, the second in that of small molecular proteins, and the third outside the protein band. The second fraction, in the present study, was purified by hydroxyapatite chromatography, with the result that FAA emerged over a narrow area of fractions. In immunoelectrophoresis and disc electrophoresis this area contained only albumin.

Key Words

Carrier proteins of folic acid
Disc electrophoresis
Folic acid fractions
Folic acid in human serum
Hydroxyapatite chromatography

According to our earlier studies, the folic acid activity (FAA) in the serum of healthy human subjects was divided, on Sephadex® G-25 gel filtration, into 2 main fractions: some 40% were inside and 60% outside the protein band [5]. Continued studies revealed that the FAA of the protein band is firmly attached to proteins. No separation was achieved either by ammonium sulphate precipitation or by repeated Sephadex filtrations. It appeared, furthermore, that the FAA of the protein band, on Sephadex G-200 filtration, was divided into 2 fractions [3]. One of these was filtered with the high-molecular proteins [4, 5] and amounted to some 5% of the total serum FAA. The other, about 30-40% of the total FAA, was filtered from the column with the small-molecular proteins [5]. This fraction will be reviewed in detail in the following study.

Material and Methods

The following phases of work were required to identify the FAA-carrying protein in the small molecular protein band: (1) gel filtration; (2) hydroxyapatite chromatography, and (3) immunoelectrophoretic examination and disc electrophoresis.

1. The gel filtration of fresh sera taken from venous blood was carried out with Sephadex G 200 without protective agents in the way described in detail previously [3]. Unlike the former method, the molarity of the sodium phosphate buffer was 0.01 M , pH 7.0. The proteins of the fractions were determined with the Beckman DU equipment at a wavelength of $280\text{ }\mu\text{m}$. The fraction volume was 10 ml. FAA was determined from the fractions as described before [3].

2. For hydroxyapatite chromatography the FAA-carrying small-molecular protein fractions obtained in phase 1 were taken apart (indicated by arrow in fig. 1). The chromatography column was prepared as follows: Bio-gel HT (Bio-Rad Laboratories, Richmond, Calif.) was suspended in 0.01 M sodium phosphate buffer and packed in this buffer into the $40 \times 1000\text{ mm}$ column. The filtration was started by washing the column with 0.01 M sodium phosphate buffer (about 100–200 ml). The elution of the proteins now took place with 0.075 M sodium phosphate buffer at pH 7.0. Fraction volume was 5 ml, and proteins and FAA were determined after this phase as described before [1] (fig. 2).

3. The FAA-carrying protein fractions obtained in phase 2 (indicated by arrow in fig. 2) were studied by immunoelectrophoresis [7, 8] and disc electrophoresis [1] to identify the proteins, as previously described in detail. The results are shown in figures 3 and 4.

Results

1. In Sephadex G 200 gel filtration the serum FAA was divided into 3 maxima: the first in the band of large molecular proteins, the second in

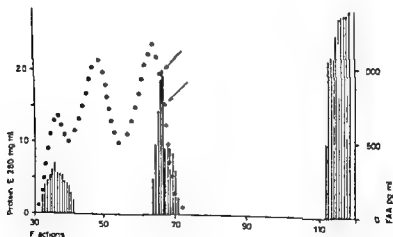


Fig. 1. Sephadex G 200 filtration of the test serum. The patient was a man aged 32.

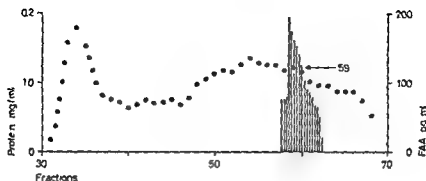


Fig 2 Continued treatment in hydroxyapatite chromatography of the protein fractions obtained on Sephadex G 200 filtration (fig 1). The protein and FAA contents are indicated in the same way as in figure 1. With the protein fractions of figure 1 14.75 ng FAA was applied to the column and the recovery was 14.00 ng FAA (95%). Fraction 59 was taken from the FAA maximum for protein identification by immunoelectrophoresis (fig 3) and disc electrophoresis (fig 4).

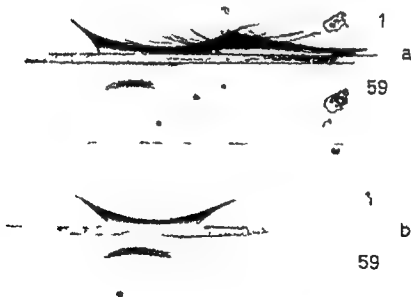


Fig 3 a Immunoelectrophoretic examination with antihuman serum of fraction 59 obtained by hydroxyapatite chromatography (1: the antisera from Behringwerke AG Marburg, FRG). b Identification of the same fraction with antialbumin (1).

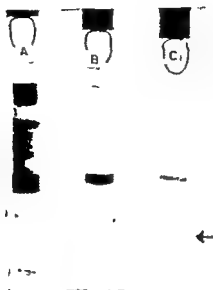


Fig 4 Disc electrophoresis (A) of the test serum, (B) of the Sephadex G-200 fraction indicated by arrow in fig 1, and (C) of fraction 59 obtained on hydroxyapatite chromatography. The last mentioned contains only pure albumin. The arrow indicates the point where the run ended.

that of small-molecular proteins, and the third outside the protein band (fig 1).

2 When the fraction of small molecular proteins was further fractionated by hydroxyapatite chromatography, all applied FAA was found in the band of the last proteins. The recovery was 95% (fig 2).

3 Immunoelectrophoresis and disc electrophoresis revealed, that the FAA-binding fractions obtained in hydroxyapatite chromatography contained only albumin (fig 3 and 4).

Discussion

In our earlier studies we have been able to show that serum FAA was divided into 3 maxima: the largest (about 60%) outside the protein band, a small portion (about 5%) in the band of large-molecular proteins, and the vast majority of the FAA of the protein band (about 30–35% of the total FAA) in the band of small molecular proteins [3–5]. As a result of the present

study, it was seen that the last mentioned FAA-carrying serum fraction was albumin. In discussing FAA in- and outside the protein band, it should be noted, however, that the external FAA may have become detached from the fractions of the protein band during filtrations. On the other hand, the FAA attached to protein fractions seems to be firmly bound, since it tolerates, e.g., an ammonium sulphate precipitation and repeated gel filtrations without abandoning its attachment to the corresponding proteins [unpublished data]. FAA recovery in these cases was nearly 100%. Similar results concerning the occurrence of FAA in- and outside the protein band have been reported by RETER and HUSKISSON [6, 7]. Their absorption studies with charcoal preparations coated with molecules of varying size suggested that the main serum folate binder is of molecular weight 70,000–120,000. Since the molecular weight of albumin is 69,000, it seems probable that the conclusion of these authors concerning the FAA-carrying protein agrees with our finding.

Before the kinetics of FAA in pathological clinical cases can be studied, the proteins to which FAA is bound in the band of large-molecular proteins must be ascertained (fig. 1). The technical solution of this problem may be very difficult, for according to preliminary studies these fractions carry several high-molecular proteins [5] difficult to differentiate. It may be pointed out, however, that FAA is firmly bound even in these fractions, for the complex withstands chemical and physical manipulations without degradation.

According to the experience obtained in our laboratory, the tripartition of FAA in the serum of a young and healthy subject is the rule. The first FAA maximum (in the large-molecular protein band) has exceptionally (though seldom) been found missing in the aged, especially women. Preliminary studies have also revealed that FAA associated with albumin was exceptionally low in folate deficiency and Addisonian pernicious anaemia. When megaloblastic tapeworm anaemia was corrected, FAA in the albumin band seemed to increase [unpublished data]. In clinical applications it is also interesting to study the effect of diseases affecting albumin metabolism on the binding of FAA to the albumin area.

Acknowledgement Our thanks are due for technical help to M. Sc. M. PUUKKA.

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The Occurrence of Homozygous Hemophilia in the Female

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Abstract A case of homozygous hemophilia in a female is reported. Her father was hemophiliac and her mother was carrier of hemophilia. She has 2 sons who are both hemophiliacs. The patient has had many bleeding episodes. The laboratory studies revealed that the patient has impaired clotting mechanism attributable only to factor VIII deficiency.

Key Words
Factor VIII deficiency
Hemophilia in the female
Karyotype in hemophilia
VON WILLEBRANDS disease

Although MACALIN [1] demonstrated the possible existence of female hemophilia, it has been thought that true hemophilia in the female could not exist because the double dose of the defective gene which might occur in the female bleeder would be lethal *in utero*. MORITA [2] was apparently the first to recognize the existence of homozygous hemophilia, in a case first presented at the Annual Congress of the Japanese Society of Hematology held in 1943. From the results of the coagulation tests and family pedigree, this case showed all the features of true female hemophilia. Recently, the authors re-investigated the same patient and the members of her family. The results are incorporated into the present paper.

Case Report

Y. T. Housewife, born July 1911

Family history Among her relatives there are a number of persons with a hemorrhagic diathesis which was transmitted by sex-linked recessive inheritance (fig. 1). The patient and her probable bleeder sister had married normal males, and they had 2 and 1 sons, respectively. These 3 boys have been recognized as hemophiliacs with severe arthropathy.

Past history Nothing relevant except for the bleeding episodes.

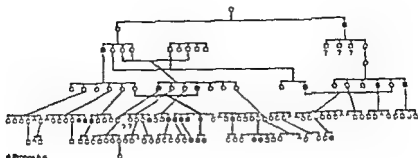


Fig. 1 Family pedigree

The course of the bleeding illness Since her early childhood, ecchymoses and intramuscular bleedings have occurred frequently and there was prolonged bleeding from minor cuts. When her deciduous teeth fell out she had severe hemorrhage especially at night. Also, many hemarthroses into the knees and ankle joints have frequently occurred since her girlhood. When she was about 16 years old these episodes were frequent and she was taken to school by car almost every 3 days despite the school was only 15 min walk away from her home. In the same year she had an episode of hematuria which lasted for several days.

Menophania was seen when she was 13 years old. At that time menstrual cycle was regular with 4 days bleeding period. The deliveries of her 2 sons, 1934 and 1936, were followed by very severe hemorrhages which caused anemia to such degree that she was almost dead. For the past 7 years she seems to be in menopause. However, bleeding episodes i.e. ecchymoses, intramuscular hematoma and hemarthroses still occur frequently.

Present status Physical examinations reveal no evidence of organic disease. She is a housewife who has grown normally with average intelligence. No anemia is present. It is observed no abnormality in her chest and abdomen except for slight hepatomegaly. There are some ecchymotic areas on both legs and on the back of the right hand. There is no severe restriction in the range of motion of her joints but muscular atrophy of right leg is clearly recognized. X-ray examinations on both knees and ankle joints show arthrosis deformans.

Methods

The bleeding time was measured by the method of DUKK. In our laboratory a bleeding time of over 5 min is considered morbid. Tourniquet test (Rumpel-Leede) was performed with the cuff of a sphygmomanometer and a positive pressure was maintained for 5 min at 10 mm under the systolic blood pressure.

Blood platelets were counted by the indirect method of MORTA and YOSHIMURA [3] (normal value $525,000 \pm 119,000/\text{mm}^3$). Blood used in the coagulation studies was procured by atraumatic venipuncture using a two-syringe method. Plasma was separated from the whole blood by centrifuging at 4°C the mixture of the blood and anticoagulant (3.8% sodium citrate) at the ratio 9 parts of blood and 1 part of anticoagulant.

Whole blood coagulation time was determined by the method of LEE and WHITE [4] (normal values 7-12 min in glass tube 20-30 min in silicone coated glass tube)

Residual prothrombin concentration in serum was measured at one hour after the coagulation was completed at 37°C by ROSENFELD and TUFT's one stage procedure [5]

Thromboplastin generation test was performed with a modified method derived from original BIGGS and DOUGLAS's method [6]

Quantitative estimations of prothrombin factor V, factor VII and X complex factor VIII were performed by WARE SEEGERS two-stage method WOLF's method [7] OWREN's method [8] and PITNEY's method [9] respectively The activity of factor IX in serum was estimated by thromboplastin generation test

The measurement of circulating anticoagulant was performed by determining recalcified clotting time when increasing amounts of the patient's plasma were added to normal plasma Plasma fibrinogen concentration was estimated by tyrosin equivalent method

Results

Blood cell count and the hemogram are shown in table I and the results of the basic coagulation studies are summarized in table II The results of the thromboplastin generation test are presented in table III Correction studies using the mixture of patient's and known hemophilic's adsorbed plasma as a reagent in the thromboplastin generation test revealed no correction of the defect (table IV) The presence of circulating anticoagulant could be excluded in reducing the clotting time to normal range by the addition of 20% of normal plasma to the patient's plasma

These results have led the authors to conclude that the patient's plasma was significantly deficient in factor VIII The number of chromosomes was 46 with normal karyotype

Discussion

The hemorrhagic tendency of the patient appeared with ecchymoses and intramuscular bleeding when she was in her early childhood Subsequently

Table I Hematological data of the patient

Hemoglobin %	90	Neutrophils stab %	20
RBC $\times 10^4$	4.90	segmented %	72.5
Colour index	0.92	Eosinophils %	1.5
Reticulocytes %	27	Basophils %	1.0
Thrombocytes $\times 10^3$	580	Monocytes %	5.0
WBC	9.100	Lymphocytes %	18.0

Table II Hemostatic studies on the patient

Bleeding time, min	2½
Rumpel Leede test	negative
Platelet count, $\times 10^3$	580
Whole blood clotting time	
in glass tube, min	24
in silicone tube, min	180
Recalcified plasma clotting time, sec	790 (166)
One stage prothrombin time, %	100
Prothrombin concentration %	95
Factor V activity, %	115
Factor VII and X complex activity, %	100
Factor VIII activity, %	<2
Factor IX activity, %	98
Residual prothrombin in serum, 1 h, %	100
Plasma clot retraction	normal
Plasma clot lysis, 24 h, 37°C	negative
Plasma fibrinogen, mg %	360
Platelet thromboplastic factor	normal

Table III Thromboplastin generation test

Thromboplastin generating mixture			Time in min that thromboplastin generating mixture was incubated before added to substrate plasma						
Source of platelets	Source of adsorbed plasma	Source of serum	1	2	3	4	5	6	7
			Clotting time in sec of substrate plasma to which thromboplastin generating mixture and CaCl_2 solution were added						
Normal	Normal	Normal	31.6	10.1	10.0	9.2	9.0	9.4	9.2
Normal	Normal	Patient	36.6	10.1	9.8	9.4	9.2	9.4	9.4
Normal	Patient	Normal	33.0	36.4	21.5	19.2	18.4	18.2	20.2
Patient	Normal	Normal	38.4	11.4	10.2	9.4	9.2	9.4	9.2

she has repeatedly had hemorrhagic episodes including persistent bleeding following cuts and hemarthroses. Laboratory observations have led to the conclusion that the substance deficient in the patient's plasma is no other than factor VIII. Anticoagulants and other inhibitory substances could be excluded.

Table II' Correction of prolonged recalcified plasma clotting time of the patient's blood by the addition of normal plasma, barium adsorbed normal plasma and known hemophilic plasma

	Mixing ratio				
Patient plasma	10	8	5	2	0
Normal plasma	0	2	5	8	10
Clotting time, sec	692	142	134	129	138
Patient plasma	10	8	5	2	0
Adsorbed plasma	0	2	5	8	10
Clotting time, sec	762	163	134	124	1,800
Patient plasma	10	8	5	2	0
Hemophilic plasma	0	2	5	8	10
Clotting time, sec	756	672	716	738	1 019

There are 2 possibilities that can give rise to a true female hemophilia. One is when a male hemophilia marries a carrier female, both contributing an X-chromosome containing hemophilic gene, the other is probably when mutation in a carrier female occurs. The former possibility was proved by BRINKHAUS [10] by presenting the female hemophilia of the dog. The authors' patient is a daughter between a hemophiliac and a carrier. And 2 sons of the patient have true hemophilia. Therefore, the patient is no doubt homozygous hemophiliac.

This case was first reported in 1943 [2]. At that time, the defective clotting mechanism and the large family pedigree definitely suggested that the patient was a female hemophiliac. It was the very first case report of a true female hemophilia. In 1951, MERSLEY [11] reported 2 cases in which the laboratory data corresponded to the data of hemophilia. In the literature there are 4 additional cases of homozygous hemophilia [12-15]. These patients, in addition to characteristic hereditary pattern, had laboratory findings showing true hemophilia, though the factor VIII assays were not performed.

Some years later, LARRIERU *et al* [16] and others [17, 18, 19] reported several patients with bleeding tendency similar to hemophilia and lacking in a plasma factor which, they thought, was factor VIII. This disease, how-

ever differs from hemophilia in several aspects. In these cases the hemorrhagic tendencies appeared with dominant hereditary pattern and the bleeding times were constantly prolonged. This condition was later referred to as von Willebrand's disease [20-21-22]. Our patient has nothing to suggest von Willebrand's disease in every sense. There are many other reports [21] on females with hemorrhagic diathesis whose blood coagulation time was prolonged. But, it is meaningless to discuss about the previously reported cases which lack the investigations which are considered necessary today.

The only point to be discussed on this patient may be to distinguish carrier state from the true hemophilia. When the reported cases of the carrier females [23-25] are reviewed it is found that they easily gave bleeding episodes and that several carriers [23-25] had partial deficiencies of factor VIII. In our laboratory 23 confirmed carriers of hemophilia A have been investigated [26]. These carriers, except for 2 cases, were healthy and without bleeding history. The exceptional 2 cases have had mild bleeding tendencies such as easy to bruise and hemorrhagia. Factor VIII concentration of the blood was reduced but significantly low while the others fell within the normal range.

In 1 reported case [31], the study of the chromosomes of a female hemophiliac showed that she was genetically male despite her physical make-up. The abortion case concerned 3 times, which proves that the case is truly female. Also the chromosome study performed for reference demonstrated a female sex-chromosome pattern.

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Prophylaxis of Joint Hemorrhages in Hemophilia¹

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Abstract A description of the continued experience acquired by transfusion of cryoprecipitates respectively 2 and 3 times a week in the prophylactic treatment of 2 young patients with hemophilia A now extending over periods of almost 3 years and more than 2½ years. The conclusion that with 3 transfusions per week of one cryoprecipitate equivalent to 900-1 500 U of AHF weekly, effective prophylaxis of joint hemorrhages is achieved in these patients seems justified. During these periods hospital admissions were reduced to a few only, and were with one exception on account of a trauma. Radiologically the changes in the joints already existing before prophylactic treatment was started showed practically no progression.

Key Words

Cryoprecipitate

Hemophilia

Joint hemorrhages in hemophilia

Transfusion in hemophilia

In a previous article [2] we described the experience acquired in prophylactic treatment of joint hemorrhages in two boys, H and K, suffering from hemophilia A who had repeated joint hemorrhages in the past, and who were in danger of becoming crippled.

Case Reports

The prophylactic treatment was carried out by transfusions of freeze-dried cryoprecipitate² prepared by the Central Laboratory of the Netherlands Red Cross. Commencing with 3 transfusions a week of respectively 1, 2 and ½-1 freeze-dried cryoprecipitates each

¹ With technical assistance from Mrs. M. L. FRIESE-VORSTELMAN. The Editors announce with great sorrow the sudden death of Prof. Dr. SIMON VAN CREVELD on March 10, 1971 at the age of 76.

² Each freeze-dried cryoprecipitate is prepared from 1 000 ml plasma. The AHF contents of 1 cryo is at the most 30-50% of AHF in 1 000 ml plasma.

prepared from 1 000 ml plasma and equivalent to a minimum of 300 U AHF, when the boys were 10 years and 10 years and 4 months old, we gradually came down to 3 transfusions a week of half a freeze-dried cryo. The latter dose was, therefore, equivalent to approximately 450-750 U AHF per week.

Prophylactic treatment of patient II was first begun on 17.11.1957, and of patient K on 10.2.1963. In a previous article [2] we reported on our findings after 10 and 8 months respectively. Since then prophylaxis was continued, and to date (1st November, 1970) covers a period of almost 3 years for patient II and more than 2½ years for patient K.

The slight complaints of various joints with restriction of movement, but not accompanied by redness or swelling of the joint, and not requiring extra transfusions which occurred when giving half a cryoprecipitate 3 times a week initially caused us not to alter the frequency of the prophylactic transfusions, but the amount of cryoprecipitate. Instead of 3 times a week ½ cryo we first gave a whole and a half cryo alternatively. We then for a while gave 3 times a week 1 cryo. Some months ago we had again come down to twice a week 1 cryo, but in view of the fact that when giving this amount temporary complaints of the joints again occurred more frequently, two months ago we went back to 3 transfusions a week of 1 cryo, equivalent to 900-1,500 U AHF per week. This was successful.

During the period following upon the one described in our previous article both patients had several injuries. These required temporary additional therapy, and on one or two occasions admission to the Clinic was necessary. (The fact that in general such injuries in hemophilia occur more frequently in childhood and during adolescence than at an adult age makes it difficult to compare the results of prophylactic treatment of children suffering from hemophilia to that of adults with the same disease.)

Complaints of the joints not only accompanied by restriction of movement, but also by slight swelling and a rise in temperature of the afflicted joint also occurred at longer or shorter intervals, particularly when we were giving 2 transfusions a week. However, by temporarily giving an extra transfusion, or by increasing the dose of cryoprecipitate for the time being these complaints quickly disappeared, and without exception much more rapidly than before when no prophylaxis was given.

When trying to find an explanation for these symptoms we should probably in addition to slight, unnoticed injuries also take into account the increased bleeding tendency in hemophilia which is often manifest at certain times of the year, as well as the differences in the AHF-contents of the various cryoprecipitates and the fact that the patients are growing. Patient II's weight increased from 30.8 kg in November, 1967 to 49.3 kg on 1.11.1970, and patient K's weight from 27.2 kg in February, 1968 to 38 kg in October, 1970.

It is of great importance that we took X rays at long intervals of several joints of both patients in which frequent hemorrhages had occurred before prophylactic treatment was started. We took X rays particularly of those joints which had given most frequent rise to complaints either or not accompanied by swelling and heat during the period of prophylactic treatment. A few of these X rays are shown in figures 1-5 (1 and 2 patient II, 3, 4 and 5 patient K.) The malformations of the joints already in existence when prophylactic treatment was begun show no further increase. From this we think we are justified in drawing the conclusion that during these years there has been no question of severe joint hemorrhages. Simultaneously the functional condition of the joints has also remained unchanged.



Fig 1 Patient II, 26.8.1968



Fig 2 Patient H, 12.11.1970



Fig 3 Patient A. 6 10 1968

Table I shows the number of admissions to the Hemophilia Clinic on account of severe hemorrhages before and after prophylactic treatment was started. The difference is self evident. In recent years the admissions were, therefore, much fewer than before and with one exception were always connected with obvious injuries.

The fibrinogen contents of both patients' plasma was repeatedly determined. This fluctuated between 330 and 420 mg%. There was therefore no question of a pathological elevation. Furthermore, the liver functions repeatedly proved to be normal, to which we should add that after the great number of transfusions of cryoprecipitates no anticoagulant developed, neither were antibodies against the Au antigen found (Dr. BRUMMELHUIS).

Moreover, the frequent transfusions continued over several years never caused unpleasant clinical symptoms. Both patients remained in excellent general condition; they



Fig 4 Patient A. 8 10 1969

both make a perfectly normal impression. They both swim and ride a bicycle. There is no longer any question of either the boys or their parents being in a state of depression which they were repeatedly before prophylaxis was started and both boys were often bed ridden on account of frequent hemorrhages.

Irrespective of those occasions on which the boys were kept at home by their parents as a precaution on account of slight complaints of the joints or on account of an obvious injury their absences from school have been insignificant. During their periods of admission to the Clinic they received regular instruction.

Discussion

Since our first article appeared there have been several more publications on the subject of longer or shorter periods of prophylactic treatment of

Table I Number of admissions in the Clinic

	Patient II	Patient III
1965	3	2
1966	2	5
1967	5	4
1968		2 once to establish prophylactic treatment, once after injury
1969	2 both times on account of injury of which once trauma capitis	2 once after injury, once for 6 days on account of a slight joint hemorrhage
1970 (until Nov 1)	1 on account of a dogbite in the left hand	1 after injury

hemorrhages in hemophilia A (and B). The starting point was as a rule the fact that in subhemophilia A in which the AITF-level of the plasma is above 5% bleeding will only occur after a more or less severe injury. Therefore, if it were possible to hold the AITF-level of the plasma above 5% a good hemostasis could always be maintained, and no more joint hemorrhages would occur. This, however, would require at least one daily transfusion of an adequate amount of AITF which in actual practice is not feasible for our patients. Moreover, those investigators who did apply such a method did not all get the same results. In our patients 24 h after transfusion of one cryoprecipitate the AITF-contents of the plasma had already descended to 2-3%, and after 48 h to the original low level.

KASPER *et al* [5] treated adults suffering from severe, classic hemophilia who had frequent hemorrhages. When they gave 250 U AITF daily the frequency of the bleeding periods was reduced to half, and with 500 U/day to 1/4 of the frequency without prophylaxis. When they gave 2,000 U once a week no bleeding occurred during the first 24 h, but did again in the middle of the week when the AITF contents of the plasma had again descended to the original low level.

BRINKHOUS *et al* [1] found that when they gave 3,000 U AITF/week divided into equal daily portions the AITF level in the plasma remained above 5% during 70% of the 24 h. During the remaining part of the period



Fig 5 Patient K, 29 7 1970

the AHF-level of the plasma was between 1-4%. When this weekly dosis of 3,000 U was given in 1 transfusion the AHF-level of the plasma remained higher than 5% for approximately only 2 days after infusion, or during 33% of the time. At the end of the period no demonstrable quantity of AHF was found in the plasma. When the 3,000 U/week were divided into equal doses given every 48 h the AHF-level of the plasma remained above 5% during the first 24 h after injection, or during 50% of the time. During the remaining period the AHF-level in the plasma was from 1-4%. No hemorrhages occurred during any of the test periods, although they were frequent when no prophylaxis was given. This experience is not quite in line with the findings of KASPER *et al* [5].

HIRSCHMAN *et al* [4] reported their experience in the prophylactic treatment of 4 patients over periods up to 2 years. Two of these patients suffered from hemophilia A. The other 2 patients were twins with the unusual combination of hemophilia A and von WILLEBRAND'S disease. When the 1st patient was given a dosis of cryoprecipitate prepared from 3 l of plasma, comparable to 1 200-1 000 U AHF, 4 times a week, no joint hemorrhages occurred during 2 months. When he was given a lower dose they did again from time to time. The 2nd patient required considerable more AHF in order to avoid spontaneous bleedings. The twins with hemophilia A combined with von WILLEBRAND'S disease had occasional joint hemorrhages when they received 4 transfusions a week, though considerably less than before.

The dosis of AHF (900-1,500 U) given weekly to our patients in 3 transfusions, when considering their weight, corresponds quite well to the doses found to give effective prophylaxis in the adult patients described above.

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R. BURKHARDT *Farbatlas der klinischen Histopathologie von Knochenmark und Knochen*. Springer, Berlin 1970 XII+115 pp., 721 pp.; DM 248.—

Die langerwünschte Erweiterung diagnostischer Möglichkeiten über die Knochenmarkszytologie, die ROHR schon 1960 praktisch ausgeschöpft hat, hinaus zur Markhistologie am unentkalkten Gewebe mit erhaltener Organotopographie ist durch die von BURKHARDT entwickelte Technik endlich wahr geworden. Der vorliegende Atlas bringt die Früchte jahrelangen Bemühens um eine verbesserte Aufarbeitung des Gewebes durch Kunststoffeinbettung, Sennünnsschnitttechnik mittels Hartschnittmikrotom und geeignete Färbemethoden, die u. a. der vertrauten May Grünwald Giemsa-Färbung nahekommen. Über 700 Farbmikrophotographien hervorragender Qualität zeugen von optimaler Strukturerhaltung des Gewebes, geben ein abgerundetes Bild des weitgespannten Spektrums von Erkrankungen des Knochenmarks und Knochens als Organ und demonstrieren überzeugend die Überlegenheit einer kombinierten zytologisch histologischen Diagnostik. Der 115 Seiten starke Band bringt zuerst eine einführende Beschreibung der angewandten Technik und kurze Übersichtsartikel über die allgemeine Histomorphologie und Pathologie sowie über die klinische Bedeutung der Histopathologie des markhaltigen Knochens (mit Verzeichnis der einschlägigen Literatur). Der Bildteil dokumentiert sodann die normale Histologie des spongiosen Beckenkammknochens, zeigt die Strukturelemente des Knochenwachstums, die normale Blutgefäßversorgung und die Elemente des retikulären Grundgewebes und des Markinterstitiums sowie des myeloischen Parenchyms. Der spezielle Teil beschreibt zuerst Panmyelopathien (mit ausführlicher Präsentation von Myelitisbildern, die der Autor offensichtlich besonders sorgfältig erforscht hat), dann Veränderungen der Erythropoese, der Thrombopoese, kombinierte Myelopoesestörungen und schliesslich die differenzierten myeloischen Hämoblastosen, das Osteomyelofibrose- und -sklerose-Syndrom sowie myeloische Neoplasien. Dabei wird bei jedem gezeigten Fall ganz kurz die klinische Symptomatologie mitgeteilt und jede Bildseite bringt — nach Art der Fussnote dargestellt — eine knappe generelle Beschreibung mit Anmerkungen zu Morphogenese, morphologischen Besonderheiten der gezeigten Bilder und mit differentialdiagnostischen Überlegungen. Auf gleiche Weise verfährt der Autor bei der Darstellung lymphatischer und retikulärer Veränderungen und schliesst mit der Demonstration der eigentlichen Osteopathien und von Tumormetastasen. Eine erwähnenswerte praktische Idee des Verfassers. Dem Band wird eine durchsichtige Schablone mit Strichplatte zum Auflegen auf die Mikrophotos mitgegeben, die die Lokalisation der jeweils beschriebenen Detailveränderungen für den Leser vereinfacht. Der Atlas ist eine willkommene Bereicherung der hämatologisch-morphologischen Literatur und ist für alle praktisch tätigen Morphologen, für hämatologisch interessierte Kliniker, aber auch für den akademischen Lehrer ein wertvoller Berater in Fragen der Diagnostik und Klassifikation. Der Immunologe, Endokrinologe oder der Onkologe findet in diesem Atlas die Illustration vieler Knochenmarksmanifestationen von Erkrankungen aus seinem Spezialgebiet und nicht zuletzt kann sich der junge lernende Mediziner mit Hilfe dieses Bandes mit der Knochenmarksmorphologie vertraut machen.

L. BIANCHI, Basel

Nucleic Acid Metabolism in Normal and Leukemic Cells

Editor E. POLLI, Milan

30 figures and 6 tables



S Karger Basel München · Paris · London · New York · Sydney

Published simultaneously in
Acta Haematologica, Vol. 45, No. 3, 1971

S. Karger Basel München Paris London New York Sydney
Arnold Böcklin-Strasse 25, CH-4000 Basel 11 (Switzerland)

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Printed in Switzerland by Buchdruckerei National Zeitung AG, Basel

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Acknowledgement

The moderator of this Symposium is deeply grateful to Professor H Lüder, who made possible the publication of the papers delivered at the Symposium on Nucleic Acid Metabolism in Normal and Leukemic Cells

The Symposium was held in Munich in 1970 at the International Society of Hematology. Recent and interesting results in the field of DNA and RNA of leukemic cells were thoroughly investigated by the researchers attending the meeting.

Introduction

E. POLLI

The progress made during these last 2 years in the study of the physico-chemical and metabolic properties of human normal and leukemic DNA concerns (1) the discovery that the chromosomal DNA of eukaryotes comprehends families of molecules having a different repetitiveness of the nucleotide sequence, (2) the study of the different molecular forms of human normal and leukemic mitochondrial DNA, (3) the discovery that DNA-polymerase, at least in *Escherichia coli*, is not the enzyme that determines DNA synthesis *in vivo*, DNA-polymerase would only play a role of repair enzyme *in vivo*.

Concerning the first point it has been shown recently by studies on the kinetics of renaturation of DNA that the genome of eukaryotes contains repeated nucleotide sequences, which are formed by short nucleotide sequences, a few hundred nucleotide long, repeated in a more or less identical way a hundred thousand times, up to one or two million times along the genome. These highly repeated fractions of the genome correspond to the nuclear satellite DNAs, which can be isolated from the corresponding main heterogeneous DNA because they band in a different position in density gradients of neutral caesium chloride or of silver and mercury-caesium sulfate at equilibrium.

Beside satellite DNAs there is in the genome of eukaryotes a fraction of DNA, having intermediate properties between those of the highly repeated satellite DNAs and of the not repeated nuclear heterogeneous DNA. This DNA, called 'the intermediate fraction' accounts in human genome for almost 20% of the total. As satellite DNAs account in humans for approximately 3-4% of the total genome, the remaining 80% is made up of not repeated heterogeneous DNA.

Concerning the function of repeated DNAs there are at present 2 main theories. According to the first, they should have a function of regulation of gene transcription. According to the second theory (the one at present supported by more experimental data) some repeated DNAs (i.e. those having the highest repetitive frequency) should have a structural role: they appear to be located in the heterochromatin in the intermitotic period and in the centromeres of chromosomes during mitotic cycle, and should have a function in the organization of chromosomes.

Regarding the second point, human mitochondrial DNA, extensive investigations have been carried out in various kinds of leukemia by VINOGRAD's group in California. The presence of circular dimers in the mitochondrial DNA of chronic myeloid leukemia has been particularly studied. This molecular form is almost completely absent in the mitochondrial DNA of normal subjects and of patients affected by other kinds of leukemia. Furthermore the frequency of circular dimers appears to be reduced following a treatment with antileukemic drugs. The interest of this finding was due to the fact that for the first time after the discovery of the Philadelphia chromosome a correlation was established between the leukemic process and a specific modification in the DNA of the leukemic cell. As it is almost certain that mitochondrial DNA codes for some structural proteins of the membranes, the presence of circular dimers in mitochondrial DNA of chronic myeloid leukemia cells could be in relation with a structural modification of the membranes. The presence of circular dimers could be a consequence of a modified activity of the enzymes which regulate DNA synthesis.

Recently it has been shown that circular dimers are present in the mitochondrial DNA of cells in tissue culture in particular conditions. They were found in tissue culture cells maintained in a stationary growth phase, or following starvation of aminoacids or treatment with cycloheximide, which inhibits cytoplasmic but not mitochondrial protein synthesis. These considerations, however, do not seem to decrease the relevance of the observation of CLAYTON and VINOGRAD.

Concerning DNA synthesis Dr. GALLO, who has been invited as a discussant, will review the argument. I would only like to point out the recent discovery that Kornberg's DNA-polymerase in *Escherichia coli* does not seem to be the enzyme responsible for DNA synthesis *in vivo*. This could perhaps open a new series of investigations on the enzymes which regulate DNA synthesis also in human leukemic cells.

For what concerns RNA, a considerable progress has been made in the study and characterization of new types of RNA: the 5 S and the 7 S,

among the RNAs having a low molecular weight. New isolation techniques, particularly the gel-electrophoresis have been acquired, by these techniques, attempts to isolate and characterize specific messenger RNAs, like those for hemoglobins and antibodies, are being made

Important progress has been made in the study of transfer RNA. It has been suggested that methylation of transfer RNA may be associated with the regulation of neoplastic transformation. Increasing hypermethylation appears to cause a progressive decrease in aminoacid acceptance. The role of aminoacyl synthetase enzymes in neoplastic induction has been investigated. I think that Dr CLINE will speak as a discussant on RNA about all these arguments, as well as about ribosomal RNA and its precursors. I would only like to make a brief comment on the giant heterogeneous nuclear RNA. This RNA, extensively studied in HeLa cells, but as far as I know not yet thoroughly studied in human leukemic leukocytes, is characterized by its high molecular weight and its nuclear localization. It could have the function of transferring a message between different sections of the genome. It might be concerned with the function of gene regulation, which has been suggested for the repeated DNAs.

E. POLLI

Nucleic Acid Metabolism in Normal and Leukemic Cells, Munich 1970
Acta haemat 45 136-158 (1971)

Synthesis and Metabolism of DNA and DNA Precursors by Human Normal and Leukemic Leukocytes

A Summary of Recent Information

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Section on Cellular Control Mechanisms Human Tumor Cell Biology Branch National
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Abstract The synthesis of DNA and the metabolism and synthesis of DNA precursor compounds in human leukocytes are reviewed. Despite the difficulties from incomplete information on leukocytes only data from leukocyte systems have been employed. Emphasis is placed on (1) control mechanisms (2) pathways and enzymes that appear to be the most relevant to chemotherapy of leukemia, (3) some of the technical problems and difficulties in interpretation of data in a heterogeneous cellular system (4) enzymatic quantitative changes during leukocyte maturation, (5) the control and alternate routes for synthesis of thymidylate the key DNA precursor, and (6) on recent information particularly regarding DNA polymerase(s).

Key Words

Chemotherapy of leukemia
DNA metabolism
Leukemic cells
Leukocyte metabolism

The task given to me by Dr. POLLER is to review the information we have on the metabolism and synthesis of DNA and some of the enzymes of DNA metabolism in normal and leukemic human leukocytes. Because of time and space limitation and my own familiarity I have in some cases utilized studies from our laboratory to illustrate specific points and I have concentrated on the most important aspects of this topic, namely, control of DNA replication.

Abbreviations used in the text: A=Adenine G=Guanine T=Thymine TdR=Thymidine (deoxythymidine) TMP or dTMP=Thymidylate (thymidine 5' monophosphate) U=Uracil UR=Uridine UMP=Uridylate dUMP=Deoxyuridylate DHT=Dihydrothymine DHU=Dihydrouracil dGTP=Deoxyguanosine-5' triphosphate dTTP=Deoxythymidine 5' triphosphate dCTP=Deoxycytidine 5' triphosphate dATP=Deoxy-

adenosine 5 triphosphate CTP=Cytidine-5-triphosphate PHA=Phytohemagglutinin
dRIP=Deoxyribose 1-phosphate 5-PRPP=5 Phosphoribosylpyrophosphate dUDP=
Deoxyuridine-5-diphosphate dCDP=Deoxycytidine 5-diphosphate

Whether one is chiefly concerned in the molecular pathogenesis of leukemias or the more practical aspects of present-day chemotherapy, cellular mechanisms for the fine control of DNA synthesis form a major focus of our interest

Some Problems in the Interpretation of Data on DNA Synthesis in Leukocytes

Before reviewing information on the metabolism and control of synthesis of deoxy nucleotides and DNA it is useful and perhaps timely to recall some of the problems inherent in these measurements, particularly applicable to leukocytes

1 *Cellular heterogeneity* In comparing any biochemical parameter between different patients, e.g., normal and leukemic it is essential to keep in mind that we are usually dealing in both cases with a mixed population of cells

2 *Cells of different levels of maturity* Since leukemic cells are mostly immature and peripheral blood leukocytes mature cells differences may only reflect differences between cells at different levels of differentiation rather than between leukemic and normal cells *per se*

To a certain degree there are some ways of getting around both these problems. First, one can isolate in relatively pure form peripheral blood lymphocytes, e.g., by nylon column chromatography, and compare these cells to CLL lymphocytes. Second, one might be interested in comparing peripheral blood of normals with those of leukemic patients in *hematologic remission*. Third, one can compare CML leukocytes with leukemoid reactions. The real problem is what normal cell do we compare to a leukemic blast cell? As yet there is no satisfactory way of obtaining sufficient quantity of pure myeloblasts or lymphoblasts from normal bone marrow. This remains one of the most important technical problems in hematology. Thus, in present, the main comparisons made between normal and leukemic 'blast' cells are by radioautography with its inherent limitations. For biochemical comparisons some approaches used in our laboratory for comparison of 'blast' cells have included (a) comparisons of ALL lymphoblasts with normal lymphoblasts obtained by PHA stimulation of normal lymphocytes [38] and (b) comparisons of the lymphoblasts that grow in long-term tissue culture originally derived from peripheral blood of patients with ALL and from normal donors [36, 65]. Although these are obviously not ideal systems, one is at least comparing cells of similar type, and in both cases the cells synthesize DNA and divide.

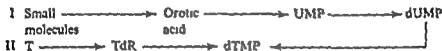
3 *Deoxyribonucleases* Leukocytes, particularly the more mature cells of the myeloid series, are rich in nucleases. Thus, to avoid artifacts, studies of the physical and chemical properties of nucleic acids (or in the case of RNA synthesis of intermediate forms) must be made with great care. This may also present a particularly formidable obstacle to obtaining data on DNA synthesis in cell free systems, e.g., assays of DNA polymerase.

The remaining problems are not peculiar to leukocytes but must be considered in any system especially when comparisons are made

4 *Variations in pool size* Consideration of differences in pool size due to different environmental conditions or different cell types is often overlooked in measurements of nucleic acid synthesis. For example, the magnitude of the increase in RNA synthesis early after PHA stimulation of lymphocytes is overestimated if the only labeled precursor used is uridine and adjustments for changes in the pool size of UMP are not made

LUCAS [51] reported that uridine kinase of lymphocytes was induced by treatment with PHA. This enzyme converts the salvage pathway nucleoside, uridine, to the nucleotide UMP. When orotic acid was used, the incorporation into RNA was not nearly so dramatically increased. Later, HAUSEN and STEIN [44], in a more comprehensive report, confirmed and extended this observation. The point is that UMP forms from two separate pathways, *de novo* from small molecules leading to orotic acid and eventually to UMP, and from uridine (salvage pathway). Hypothetically, we might say that at any given time 10 nmoles of UMP exist in a cell, 5 formed from the *de novo* pathway and 5 from uridine. Total incorporation into RNA per unit of time is 10. With an induction of uridine kinase, 8 nmoles of UMP may come from uridine but the amount from the *de novo* pathway may diminish, e.g., to 2, so that the total UMP remains 10 nmoles. However, with uridine as the labeled precursor for measurement of RNA synthesis, an increase in cpm incorporated into RNA would be found simply due to the increase in specific activity of UMP, leading to the spurious conclusion that total RNA synthesis had increased.

The same problem exists with measurements of DNA synthesis when labeled deoxythymidine (thymidine, TdR) is used as the precursor, since again two pathways exist for formation of thymidylate (dTMP), the *de novo* (I) and the salvage (II), as shown in very abbreviated form



It is likely in some cases that DNA synthesis is overestimated with TdR as the labeled precursor due to induction of TdR kinase. It is also likely that differences in DNA synthesis reported between different cell types may in some cases be due to cellular differences in these relative contributions to dTMP. That pool effects have not been adequately emphasized is apparent from the failure in a number of reports to consider gross pool changes on data relating to DNA (or RNA) synthesis, as for example, reports on inhibitory effects of whole RNA or DNA (or nuclear components) on RNA and DNA synthesis as determined by labeled uridine and TdR incorporation without cellular morphologic confirmation such as a decrease in the mitotic index.

5 *Degradation of the labeled precursor* The concentration of labeled precursor, e.g., TdR, is an important factor for measurement of DNA synthesis. High concentrations may inhibit DNA synthesis by interfering with a number of enzymatic steps, and cell death may even occur. On the other hand, too low concentrations may give artifactual differences in DNA synthesis due to a limitation in the availability of TdR, e.g., from differences in the amount of TdR phosphorylase. This enzyme can catalyze synthesis of TdR from T and a sugar (deoxyribosyl) donor [28, 31, 32, 35] but also degrades TdR [28, 33, 54, 72] the equilibrium favoring degradation. This enzyme varies in different types of leukocytes [33, 54, 72] and with TdR limiting may be responsible for marked differences in counts incorporated into DNA between different cells when, in fact, no real difference exists [see more detailed discussion in previous review, ref. 5].

6 *Replication vs repair* Incorporation of a labeled DNA precursor molecule into DNA does not mean that actual replication of DNA has taken place. Alternatively, incorporation may reflect repair synthesis involving only small segments of the DNA.

The following approaches in our laboratory to diminish problems with points 3 and 4 are (a) use of more than one label, e.g., for DNA synthesis, TdR, ^{32}P , and orotic acid, (b) pool determinations; (c) supporting morphological data, e.g., mitotic index

'Levels' of Control of DNA Synthesis

There are at least 4 major areas for examining the control of DNA synthesis: 1. Control of the supply of essential precursors, particularly thymidylate. 2. Regulation of the activity of the physiological DNA replicating enzyme(s). 3. The interplay of factors that govern the physical state of the DNA primer. 4. The regulation of the amount and the localization of 'initiator' and 'repressor' molecules. This discussion will deal with only the first 2 of these areas.

Control of the Supply of Pyrimidine Deoxynucleotides

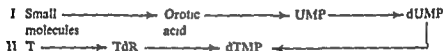
Although precursor control of DNA or RNA synthesis is increasingly being viewed as a coarse rather than a fine control, nonetheless it remains an area of great importance since some of the most effective anti-tumor agents appear to work at this level. In any event, it is at this level of control that we have the greatest amount of information on leukocytes. The essential reactions of pyrimidine nucleotide biosynthesis and catabolism which have at least been partially verified in leukocytes are presented in figure 1.

From small molecules carbamyl phosphate is synthesized. These reactions have been well studied and reviewed elsewhere [24]. However, in leukocytes the mechanism of carbamyl phosphate formation has not been clearly demonstrated. For this reason in figure 1 the *de novo* biosynthesis of the pyrimidine ring begins with carbamyl phosphate. In leukocytes the series of reactions leading to formation of the first pyrimidine nucleotide, orotidylate (OMP), has been studied chiefly by SMITH and BAKER [76] and by SMITH *et al* [78, 79]. An important point about this portion of pyrimidine biosynthesis is that feedback control of aspartate transcarbamylase, the enzyme catalyzing reaction 1, has not been demonstrated in human leukocytes [62, 77]. This protein is an allosteric enzyme in *E. coli* with a regulatory subunit, allowing for feedback control.

normal and CML leukocytes was found. Thus, in human leukocytes the mechanism of control of the early enzymatic activities remains to be shown. It is important to bring this out again since even in recent texts the concept that normal cells but not CML cells have feedback control of pyrimidine nucleotide formation was again quoted. A more detailed discussion of leuko-

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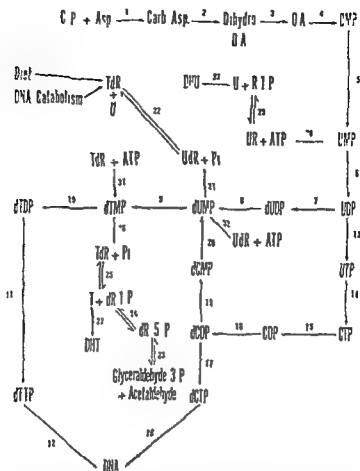


Fig 1 Pyrimidine deoxynucleotide synthesis, metabolism, and interconversions. The numbers refer to specific enzymes. Reactions 1-4 lead to the formation of the pyrimidinering. Enzyme 1 is the allosteric enzyme aspartate transcarbamylase, 5, orotidylate decarboxylase, 7 and 16, ribonucleotide reductase, 11 and 13, undylate kinase, 9, thymidylate synthetase, 10 and 11, thymidylate kinase, 12 and 18, DNA polymerase, 20, deoxycytidylate deaminase, 21 and 26, pyrimidine deoxynucleotidases (phosphatases), 23, deoxyriboaldolase, 24, deoxyribosephosphomutase, 27 and 30, dihydrouracil and dihydrothymine dihydrogenase, 22, pyrimidine deoxyribosyltransferase, 25, thymidine phosphorylase, 31 and 32, thymidine (deoxyuridine) kinase, 28, uridine kinase, 29, uridine phosphorylase. Abbreviations: CP = carbamyl phosphate, Carb Asp = carbamyl aspartate, Dihydro OA = dihydroorotic acid, OA = orotic acid. Other abbreviations are described in the footnote on page 136.

[From ref. 58, p. 1252, with the permission of the publishers.]

cyte regulatory (allosteric) enzyme has been recently presented elsewhere [58].

Since the thymine nucleotide (instead of uracil) and deoxyribose (in place of ribose) are the only components of DNA that distinguish it from RNA,

the mechanisms for their immediate synthesis are of particular interest. In addition, some anti-tumor agents are thought to exert their effects at these levels.

Thymine Nucleotide Synthesis

Reaction 9 of figure 1 illustrates one mechanism for the formation of dTMP, the conversion of dUMP to dTMP. A considerable amount of information on this reaction in leukocytes is available, particularly from the contributions of BERTINO *et al* [8-10], SILBER *et al* [74, 75], HULTIN-KRUM *et al* [47], and ROBERTS and HALL [66, 67]. The reaction, of course, requires two enzymes, dTMP synthetase and dihydrofolate reductase. The former is inhibited by FUdR and the latter by methotrexate. However, studies on resistance of leukemic cells to methotrexate leave some doubt that this is the only site of its action.

The second mechanism for direct synthesis of dTMP is by phosphorylation of TdR catalyzed by TdR kinase (reaction 31 of figure 1). This reaction and the subsequent further phosphorylation of dTMP to dTDP and dTDP to dTTP (catalyzed by dTMP kinase, see reactions 10 and 11 of fig. 1) have been characterized and quantitated in leukocytes, particularly by BIANCHI [11], and by BIANCHI *et al* [12], and by NAKAI *et al* in CRADDOCK's laboratory [55]. This salvage pathway enzyme may be increased following inhibition of the *de novo* formation of dTMP, e.g., after aminopterin, and this increase could be an important reason for development of resistance to the cytotoxic effects of this agent by bypassing the cellular requirements of dTMP from dUMP.

The relative contribution of the salvage pathway enzyme (TdR kinase) compared to the *de novo* pathway for dTMP formation in leukocytes has not been completely clarified, although considerable information has been provided, particularly by the studies of COOPER *et al* [20, 21]. There are a number of reasons to believe that TdR kinase plays an important role when growth is stimulated [cf. ref. 58 for a detailed discussion]. It is known that if the contribution to dTMP from the salvage pathway is increased, e.g., by increasing intracellular TdR, the formation of dTMP from the *de novo* pathway is *not* inhibited, at least in CML leukocytes [20]. This has not been verified in normal cells. Does this mean that increasing intracellular TdR should in itself lead to DNA synthesis by increasing the availability of dTMP? We know of no direct studies which indicate that this is the case, which indicates that although the supply of dTMP might be a factor limiting the rate of DNA synthesis and its formation is clearly favored under growth stimulating

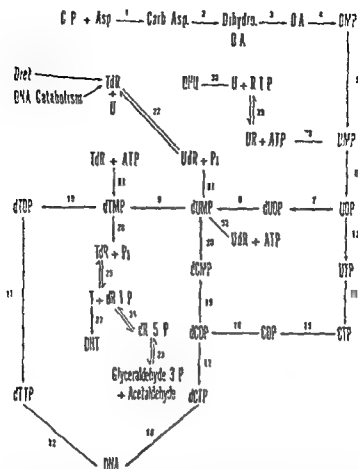


Fig 1 Pyrimidine deoxynucleotide synthesis, metabolism and interconversions. The numbers refer to specific enzymes. Reactions 1-4 lead to the formation of the pyrimidinering. Enzyme 1 is the allosteric enzyme aspartate transcarbamylase, 5, orotidylate decarboxylase, 7 and 16, ribonucleotide reductase, 6 and 13, uridylyate kinase, 9, thymidylate synthetase, 10 and 11, thymidylate kinase, 12 and 18, DNA polymerase, 20, deoxycytidylate deaminase, 21 and 26, pyrimidine deoxynucleotidases (phosphatases), 23, deoxyriboaldolase, 24, deoxyribosephosphomutase, 27 and 30, dihydrouracil and dihydrothymine dihydrogenase, 22, pyrimidine deoxyribosyltransferase, 25, thymidine phosphorylase, 31 and 32, thymidine (deoxyuridine) kinase, 28, uridine kinase, 29, uridine phosphorylase. Abbreviations: CP = carbamyl phosphate, Carb Asp = carbamyl aspartate, Dihydro OA = dihydroorotic acid, OA = orotic acid. Other abbreviations are described in the footnote on page 136.

[From ref. 58, p. 1252, with the permission of the publishers.]

cyte regulatory (allosteric) enzyme has been recently presented elsewhere [58].

Since the thymine nucleotide (instead of uracil) and deoxyribosyl (in place of ribosyl) are the only components of DNA that distinguish it from RNA,

of intracellular dTMP. As would be anticipated, alkaline phosphatase activities are highest in fully mature leukocytes where no DNA synthesis occurs and least in the blast cells where DNA synthesis occurs. In fact blast cells were thought not to contain any alkaline phosphatase as determined by a histochemical assay [84]. However, this may be not the case, as shown in figure 3. Thymidylate, dTMP (abbreviated TMP in the figure), is metabolized by intact leukemic human lymphoblasts. ^3H -dTMP ($5\ \mu\text{M}$) was added to the cell suspension and aliquots of the media were analyzed at the designated intervals for disappearance of dTMP and appearance of labeled TdR, T, and the breakdown product of T, dihydrothymine (DHT). The dTMP is very rapidly converted into TdR, virtually all of it is metabolized within 1 h and the majority within 10 min. The appearance of TdR correlates with the decline in dTMP. As TdR itself is metabolized, T, and still later DHT, appear in the media. The remaining counts are incorporated into DNA as ^3H TdR is reconverted by TdR kinase and ATP back to ^3H -dTMP in the cell.

The available evidence suggests that most nucleotides are not taken up or minimally taken up by mammalian cells. How then was exogenous dTMP metabolized? Does exogenous dTMP get incorporated directly into DNA? We analyzed this question by the following experiment. Lymphoblasts from a patient with ALL were incubated with media containing 2 mM phosphate buffer and either $5\ \mu\text{M}$ ^3H -dTMP or $5\ \mu\text{M}$ dTMP P^{32} (labeled). The media was analyzed at intervals for the appearance of inorganic phosphate (^{32}P -Pi) and the DNA of the cells isolated for measurement of the incorporation of either ^{32}P or ^3H labeled dTMP. The data are shown in figure 4. No incorporation of dTMP- ^{32}P was found while very significant incorporation of ^3H -dTMP took place. Furthermore, inorganic phosphate appeared in the media almost parallel with the incorporation of ^3H counts into DNA. From these findings we can conclude that exogenous dTMP is not incorporated into DNA, but as illustrated also in the previous experiment (fig. 3), it is rapidly dephosphorylated to TdR. The TdR in turn is then converted in the cell back to dTMP and incorporated into DNA. Apparently, dTMP cannot enter the cell but is probably metabolized at the membrane to form TdR, which is readily taken up by the cell and converted back to dTMP intracellularly.

After a Growth Stimulus Changes in Pyrimidine Metabolism Favor Formation of dTMP

Following a stimulus to growth (e.g., partial hepatectomy, serum addition to contrast inhibited tissue culture cells, PHA stimulation of 'resting' lym-

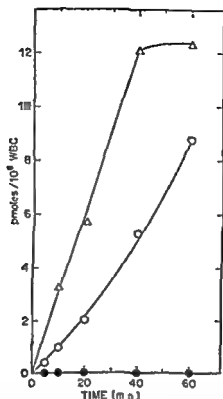


Fig 4 Comparison of the incorporation of base labeled ^3H TMP (Δ) and phosphate labeled TMP ^{32}P (\bullet) into human lymphoblast DNA. Cells were incubated in media with 2 mM phosphate buffer and labeled TMP. Aliquots obtained at the designated intervals and (1) the media was analyzed for the appearance of labeled inorganic phosphate (^{32}P -Pi, and (2) the cellular DNA isolated and the incorporation of counts determined. No ^{32}P TMP was incorporated into DNA, in contrast, counts from ^3H base labeled TMP were incorporated. The results indicate that intact TMP does not enter the DNA but is metabolized at the membrane to form TdR. The latter in turn enters the cell to reform TMP which is then incorporated into DNA.

[From ref. 58, p. 1259, with the permission of the publishers.]

phocytes, etc.), several biochemical changes occur which favor formation of dTMP to meet the cell's requirements for DNA synthesis. The salvage pathway contribution to dTMP formation increases by the following mechanisms: (a) synthesis of dR-5-P from glyceraldehyde-3-P and acetaldehyde increases (see reaction 23, fig. 1) [14]. By conversion of dR-5-P to dR-1-P (deoxyribosephosphomutase) dR-1-P may also increase. Concomitantly, intracellular T may increase since its catabolism to DHT is diminished [16]. With an increase in intracellular T and dR-1-P, TdR synthesis (catalyzed by TdR phosphorylase) is favored. Furthermore, increase in T inhibits TdR degradation by

phosphorolytic cleavage, further favoring TdR formation. The increase in TdR should be very effectively utilized since with a growth stimulus the activity of TdR kinase is also enhanced [13, 85]. (b) the formation of Udr should be favored by the same mechanisms favoring TdR synthesis. Furthermore, Udr is also a substrate for TdR kinase ($\text{Udr} + \text{ATP} \rightarrow \text{dUMP} + \text{PP}_i$). Thus dUMP formation is also favored and since a stimulus to growth has also been reported to enhance dTMP synthetase ($\text{dUMP} \rightarrow \text{dTMP}$) [17] a second mechanism for increasing dTMP is available. Finally, the catabolism of dTMP by 5-nucleotidase was reported to be diminished after a growth stimulus [26].

Another mechanism for possible enhancement of dTMP formation (from dUMP) is by enzymatic deamination of dCMP to dUMP. This enzyme, deoxycytidylate deaminase, has interesting allosteric properties, and has been characterized extensively by SCARANO *et al* [70] and later in human leukocytes by SILBER [73] and its activity is also enhanced by a stimulus to growth [53].

Implications for Chemotherapy of the Metabolic Interrelationships of Pyrimidine Deoxynucleotides and Their Alteration with Growth

One of the key targets for inhibition of cell proliferation has been to 'starve' the cell of the essential DNA precursor dTMP. This may be achieved for example, with 5-FU, 5-FUdr, methotrexate, and BUdr. Resistance to chemotherapy is a complex phenomenon which may involve many mechanisms. In view of the alternate paths for dTMP formation and the interrelationships of dTMP precursors it is apparent that inhibition of one pathway although perhaps initially effective, might eventually result in compensatory increases in other pathways. It is not difficult to envision just from this point of view the advantage of present day combination chemotherapy over single agent chemotherapy, particularly when anti metabolites are used. In an acute blastic phase of leukemia with more rapid cell proliferation, an increase in the mechanisms operative for favoring deoxynucleotide biosynthesis probably occurs as illustrated in the preceding section for dTMP. Thus in these clinical situations if anti metabolites alone were used, the amount and number required to kill a given number of cells might be substantially increased.

The Use of In Vitro Assays of 'DNA Synthesis' for Estimating the Clinical Efficacy of Anti Leukemic Agents

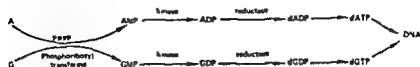
In recent years there have been some reports of laboratory tests designed to estimate the efficacy of anti tumor agents. They have primarily consisted

of measuring the effect of a given agent on 'DNA synthesis' by determining the incorporation of labeled TdR into leukocyte DNA *in vitro*. The purpose of this approach is to help individualize chemotherapy by obtaining predictive information. Thus, the effect of different agents on DNA synthesis of leukocytes obtained from individual patients might be a guide for the clinical response of the patient to the drug in question. However, the problems in interpretation of these results are formidable. For instance, by inhibiting formation of dTMP from dUMP by methotrexate a true inhibition of DNA synthesis might occur because of limiting supply of dTMP. However, the use of TdR as the labeled precursor may give any one of 3 results: (1) inhibition (reflecting the true diminution of DNA synthesis), (2) increased incorporation of TdR because of a greater specific activity of labeled dTMP (due to a decrease in the pool of unlabeled dTMP more counts are incorporated but the reduction of total available dTMP is significant enough that DNA synthesis is, in fact, reduced), (3) increased incorporation with a true increase in DNA synthesis. The decrease in dTMP from methotrexate inhibition of TMP synthetase may be more than compensated for by an induction of TdR kinase, the result being that the pool of dTMP is normal. If an increased TdR incorporation was found in this case, it would really reflect the true status of DNA synthesis in the leukocytes of this patient. To complicate matters more, differences exist among cell types and between different patients on the rate of TdR catabolism. Thus TdR may become limiting in some cases and give a false low value for DNA synthesis. Finally, the mechanism by which resistance develops to drugs may involve metabolism by tissues other than leukocytes. It is not surprising then that these types of tests have not gained widespread use, and it is doubtful that they will have any real practical usefulness as predictive tests for response of a given patient to chemotherapy.

Control of the Supply of Purine Deoxynucleotides

As with pyrimidines, the biosynthesis of purine deoxyribonucleotides may proceed from two alternate routes, *de novo* and 'salvage' pathways. However, it has been indicated that leukocytes have little capacity for formation of the purine ring *de novo* [48, 71]. This is remarkable for a proliferative cell, and for leukocytes the salvage pathways for purine nucleotide and deoxynucleotide formation would appear to have greater importance. The work of LAJTHA and VANE [48] suggests that the purine bases may in part be derived from the liver. Another source may, of course, be from nucleic acid catabolism. In either case the formation of the final immediate purine DNA precursors purine deoxynucleoside triphosphates, would occur via the salvage pathways.

I HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE + RIBONUCLEOTIDE REDUCTASE



II PURINE DEOXYNUCLEOSIDE PHOSPHORYLASE + KINASES

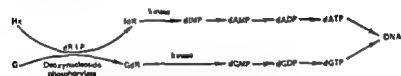


Fig 5 Probable major pathways for purine deoxynucleotide synthesis in leukocytes. Both pathways utilize preformed purine bases (salvage pathways) which apparently are derived from other organs and from DNA catabolism.

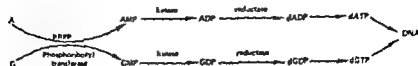
These pathways are illustrated in figure 5. There are at least two possible purine deoxynucleotide salvage pathways. The more important pathway appears to be the direct synthesis of the ribonucleotide from a purine base and phosphoribosyl pyrophosphate (PRPP). The nucleoside monophosphate is then converted to the diphosphate by a purine nucleotide kinase and, as in the case of pyrimidines, the deoxyribonucleotide is formed at the diphosphate level via ribonucleotide reductase. The purine phosphoribosyltransferases in leukocytes have been studied by LUGANOVA and SEITZ [52] and by DAVIDSON and WINTER [25]. As expected, this enzyme activity is higher in leukemic leukocytes compared to normal leukocytes from peripheral blood. It has been suggested that one of the mechanisms for resistance of tumor cells to 6-mercaptopurine (6-MP) may be by loss of this enzyme activity with the result that the active form, 6-MP nucleotide, would not be synthesized. However, if that is so, and if this is the main pathway for purine nucleotide synthesis, how does the resistant tumor cell continue to live? A second 'salvage' pathway studied in our laboratory also exists in leukocytes. This is shown in the lower part of figure 5. Here, instead of direct formation of the ribonucleotide, the purine base, by reacting with deoxyribose 1-P forms the respective deoxynucleoside. The latter are converted to deoxynucleotides by purine deoxynucleoside kinases. This pathway then differs from the other in (a) requiring a deoxynucleoside intermediate, (b) not requiring a ribosyl intermediate,

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E. HYPOXANTHINE GUANINE PHOSPHORIBOSYLTRANSFERASE + RIBONUCLEOTIDE REDUCTASE



II. PURINE DEOXYNUCLEOSIDE PHOSPHORYLASE + KINASES

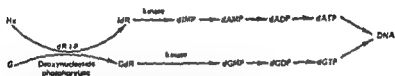


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of measuring the effect of a given agent on 'DNA synthesis' by detecting the incorporation of labeled TdR into leukocyte DNA *in vitro*. The use of this approach is to help individualize chemotherapy by obtaining more information. Thus, the effect of different agents on DNA synthesis in leukocytes obtained from individual patients might be a guide for the response of the patient to the drug in question. However, the proper interpretation of these results are formidable. For instance, the inhibition of formation of dTMP from dUMP by methotrexate is a true inhibition of DNA synthesis might occur because of limiting supply of dTMP. The use of TdR as the labeled precursor may give any one of 3 results: (1) no incorporation (reflecting the true diminution of DNA synthesis), (2) incorporation of TdR because of a greater specific activity of TdR (due to a decrease in the pool of unlabeled dTMP more counts are incorporated but the reduction of total available dTMP is significant and DNA synthesis is, in fact, reduced); (3) increased incorporation with no change in DNA synthesis. The decrease in dTMP from methotrexate inhibition of TMP synthetase may be more than compensated for by an increase in thymine kinase, the result being that the pool of dTMP is normal. If no incorporation was found in this case, it would really reflect a decrease in DNA synthesis in the leukocytes of this patient. To complicate matters, differences exist among cell types and between different patients in the rate of TdR catabolism. Thus TdR may become limiting in some cases, giving a false low value for DNA synthesis. Finally, the development of drug resistance in leukocytes may involve metabolism of the drug. It is not surprising then that these types of tests have a limited usefulness as predictive tests for response of a given patient.

Control of the Supply of Purine Deoxynucleotides

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(b) *RNA-dependent DNA polymerase* The recent unique observations of TEMIN and MIZUTANI [82] and BALTIMORE [4] that RNA oncogenic viruses synthesize DNA from an RNA template, thus providing a mechanism for reversal in the 'flow' of genetic information, gave dramatic new insight into how an RNA virus might introduce a stable 'lesion' in an animal cell, i.e., how the characteristic (neoplastic growth) could be passed from one cell generation to the next without the virus containing DNA. This finding was rapidly confirmed and extended with several RNA oncogenic viruses in many laboratories, but in particular by SPIEGELMAN *et al* [80, 81], GREEN *et al* [42], ROKUTANDA *et al* [68], and GERWIN *et al* [41]. Recently, we reported the finding of an analogous enzyme in human acute leukemic cells [37, 38] and showed that within limits of sensitivity of our assay the enzyme was not detected in normal peripheral blood lymphocytes. The enzyme, which was partially purified, was inhibited by N-demethylrifampicin, a derivative of the antibiotic rifampicin. In contrast, DNA dependent DNA polymerase from normal lymphoblasts was not significantly inhibited [37]. Is this the enzyme of one of the RNA oncogenic viruses and if so, will inhibition of the enzyme have a direct anti tumor effect or perhaps more logically reduce the development of relapse after remissions have been obtained? If the enzyme is in normal tissues as suggested by the subsequent finding of an activity with the DNA-RNA hybrid poly dT-rA with established normal tissue culture lines¹, does it function to amplify genes for specific RNAs in cell differentiation? These are some of the critical questions that remain to be answered.

DNA Repair Enzymes

The repair of chemically or physically altered DNA (repair replication) involves a break in the chain, followed by removal of the damaged region, insertion of new bases, and then 'closure' [43]. Although a great deal more needs to be learned, presumably these steps require different enzymes, a specific endonuclease, the repair replicating enzyme, and polynucleotide ligase. Fibroblasts from patients with xeroderma pigmentosum have a defect in the early process of repair, apparently the endonuclease which recognizes the

¹ While this manuscript was in preparation SCOLNICK *et al* [SCOLNICK, E. M., AARONSON, S. A., TODARO, G. J., and PARKS, W. F. RNA-dependent DNA polymerase activity in mammalian cells. *Nature, Lond.*, 229: 3181 (1971)] reported detecting an enzyme activity with the DNA-RNA hybrid.

damaged region and breaks the polynucleotide chain [19]. There is no evidence for a defect in these enzymes in human leukemia.

Summary of Comparative Results of Enzymes Involved in Metabolism and Synthesis of DNA in Normal and Leukemic Leukocytes

A great many quantitative differences can be found in the activities of different enzymes from normal and leukemic leukocytes. In the vast majority of cases these differences simply reflect differences in degree of cellular maturation. As leukocytes differentiate the quantity of a great many protein changes, e.g., the enzymes involved in the biosynthesis of deoxyribonucleotides and DNA diminish and in some cases cannot be detected by the time a mature granulocyte has formed. Concomitantly, there is an increase in 'mature cell protein', i.e., enzymes involved in the function of the mature leukocyte, e.g., the hydrolases of granulocytes take part in 'digestion' of material taken in during phagocytosis. While DNA biosynthetic enzymes diminish, deoxyribonuclease activity, for example, is increased. Thus, many enzyme differences are expected when leukemic blasts are compared to normal peripheral blood leukocytes which, of course, consist primarily of mature granulocytes. Even when comparisons are made between leukemic blasts and leukemoid reactions, differences may occur due to differences in degree of cellular maturity rather than leukemia *per se*.

In a few cases quantitative enzyme differences have been found between normal and leukemic cells, even though the degree of maturation appears to be the same by morphological criteria. These include (1) alkaline phosphatase, which is by far the most extensively documented enzyme difference between a leukemic and normal leukocyte. The initial observations 20 years ago by BECK and VALENTINE [6] showed that leukocytes of patients with chronic myelocytic leukemia (CML) have diminished or have non-detectable activity. Even when patients with CML are in complete hematological remission with a normal number of morphologically normal mature granulocytes, the level of this enzyme may be reduced, (2) pyrimidine deoxyribosyl transferase was also found to be reduced in CML leukocytes compared to normal leukocytes, even when the CML patients were in remission with morphologically mature granulocytes [34], (3) DNA polymerase, which according to OVE *et al* [57] is higher in cells from CML patients (not in remission) than in immature normal cells. In this case the activities were not determined in normal mature granulocytes and mature granulocytes from CML patients in remission.

I would still attribute the differences in these enzymes to comparisons of

cells that are not of identical maturity, i.e., even though morphology suggests that the level of maturity is the same, these enzyme differences may indicate that biochemical results are a more sensitive index of the degree of maturation than morphology. A possible exception to this (at least quantitatively) may be the RNA dependent DNA polymerase which has been found in leukemic leukocytes in our laboratory [37, 38] and by SPIEGELMAN *et al* [personal communication and from his presentation at the 2nd Annual Lepetit Symposium Paris 1970]. Usually no detectable activity or lower activity has been found in proliferating controls. However, more studies are needed before this is established.

In no case has a qualitative enzyme difference been demonstrated between normal and leukemic cells. Physical and chemical properties such as pH optima, thermal stability, K_m 's, K_i 's, molecular weights, and regulation by metabolites, substrates, analogues, etc., have been identical in every case where these types of enzyme comparisons have been made.

Mitochondrial DNA

During the last few years it has been shown that the mitochondria of animal cells contain DNA which is metabolically active [3] and which consists of several structural forms [18, 46]. One structural form, a circular dimer (i.e., a circle of DNA twice the length of the 5 μ m monomer) was discovered by CLAYTON and VINOGRAD in CML leukocytes but not normal cells which lead to a proposal that this might be contributory to the oncogenic process [17]. However, NASS later showed that by altering growth conditions, these dimers could be regularly produced in L-cells, which under usual conditions do not contain the dimers [56].

The State of the DNA Template in Control of DNA Synthesis

The physical state of the DNA primer may be critical to the control of the initiation and rate of DNA synthesis, but there is little information on this in leukocytes. The reports by POLI *et al* and by POLI and SHOOTER indicate that some physical properties of DNA from leukemic leukocytes differ from normal leukocytes [59, 60]. These differences might provide future leads to an understanding of why DNA of leukemic cells remains 'active' while normal leukocyte DNA progressively loses its ability to replicate during maturation.

Rates of DNA Synthesis and Mitosis

Despite limitations and difficulties in interpretation of comparative data on rates of nucleic synthesis of normal and leukemic cells, a number of

observations over the last decade have reasonably established that leukemic cells are not the 'wildly' proliferating cells commonly referred to. Thus, leukemic cells apparently do not synthesize DNA or divide faster than normal blast cells. In fact, if anything, synthesis of DNA may be reduced. This idea, derived particularly from the studies of ASTALDI and MAURI [1, 2], GALOSTO *et al* [39, 40], CRADDOCK and NAKAI [22], and CROVATE *et al* [23] appears to me to have been one of the most important concepts affecting the development of new thoughts on human leukemogenesis and therapy.

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Regulation of Deoxyribonucleotide Synthesis by Ribonucleotide Reductase in Leukemic Leukocytes

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Abstract A 13-fold increase was observed in the specific activity of ribonucleotide reductase in the spleen of mice injected with a murine leukemia virus. There was an absolute requirement for a hydrogen donor, dithiothreitol. The ribonucleoside diphosphates were preferred substrates. The reduction rate was affected by the presence of nucleoside triphosphates. Ribonucleotide reductase is present in acute and chronic myelocytic leukemia leukocytes. Normal and chronic lymphocytic leukemia leukocytes showed no significant activity. Coenzyme B₁₂ does not stimulate the enzyme from megakaryoblastic bone marrow.

Key Words

DNA metabolism
Leukemic cells
Mouse leukemia
Ribonucleotide reductase

Regulation of the enzymatic reduction of ribonucleotides to deoxyribonucleotides has been investigated in several microbial [1-3] and animal systems [4-7]. This reduction is catalyzed by ribonucleotide reductase and is generally regulated through the concentrations of nucleotide, which act as allosteric effectors [8-11]. The fact that deoxyribonucleotides are present only in very small amounts in cells suggests that the reduction of ribonucleotides to deoxyribonucleotides would be a suitable locus for the regulation of DNA formation [5, 12]. In view of the probable importance of this reaction in cell proliferation, and the potential value of this reaction as a target site for antimetabolites [13, 14], it seemed pertinent to determine the levels of the enzyme and regulation of this system in the course of viral leukemogenesis and in the human leukemic leukocytes.

Materials and Methods

Female adult Swiss mice were inoculated intraperitoneally with Friend murine leukemia virus. Group of 6-10 animals were sacrificed at various time intervals. The enzyme was

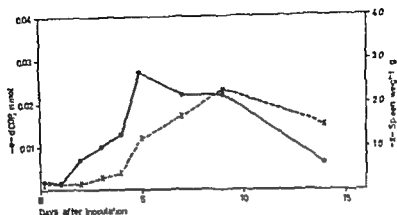


Fig. 2 Specific activity of ribonucleotide reductase in mouse spleen after infection with murine leukemia virus [7].

Table 1 Purification of ribonucleotide reductase from mouse leukemic spleen [7]

Step	Volume ml	Protein mg	Total activity μ mol	Specific activity μ mol mg
Crude extract	12	840	11.8	0.014
Sephadex G-25 chromatography	25	675	44.6	0.066
1% Streptomycin sulfate supernatant	24	504	56.5	0.112
25-40% Ammonium sulfate precipitate	2.5	29	29.3	1.029

The specific activity of ribonucleotide reductase in the spleen following infection with murine leukemia virus is shown in figure 2. On the second day after inoculation, the specific activity had risen to over 3 times that of the control. Over the next 3 days, there was a sharp increase in the enzyme level to a peak specific activity approximately 13 times higher than the average for normal spleen. Thereafter, a gradual decline occurred.

The purification of ribonucleotide reductase from leukemic spleen is summarized in table 1. After ammonium sulfate precipitation, the enzyme became very labile. Attempts to stabilize the enzyme with 2-mercaptoethanol,

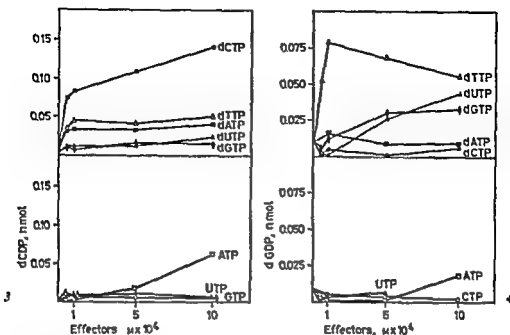


Fig 3 Effects of deoxyribonucleoside triphosphates and ribonucleoside triphosphates on CDP reduction [7]

Fig 4 Effects of deoxyribonucleoside triphosphates and ribonucleoside triphosphates on GDP reduction [7]

dithiothreitol, ascorbic acid, 10% glycerol, EDTA, Mg^{++} , Ca^{++} , or phenylmethyl sulfonyl fluoride were without effect. dCTP and ATP did not stabilize the enzyme for storage or purification. Precipitation of the enzyme at pH 5.0 resulted in a 75% loss in specific activity.

The reduction by ribonucleotide reductase is generally influenced by the presence of different nucleotides. A comparison of the effects of different nucleoside triphosphates on CDP reduction is shown in figure 3. The addition of dCTP and ATP resulted in marked stimulation of the reaction, dTTP and dATP were less effective than dCTP and ATP. Figure 4 shows the effects on GDP reduction. GDP reduction was greatly increased by the addition of dTTP. Optimal activity was observed at a dTTP concentration of 10^{-4} M. dUTP and dGTP showed lower stimulatory effects.

Allosteric activators of ribonucleotide reductase from *E. coli* [8], rat Novikoff hepatoma [9], and mouse leukemic spleen are compared in table II. Activator's specificity of ribonucleotide reductase from mouse leukemic spleen closely resembles that of *E. coli* and Novikoff hepatoma. Exception

Table II Activators of ribonucleotide reductase from different sources

Enzyme source	Substrate			
	CDP	UDP	ADP	GDP
<i>E. coli</i>	dTTP ATP	dTTP ATP	dTTP dGTP	dTTP dGTP
Rat Novikoff hepatoma	ATP	ATP	dGTP	dTTP
Mouse Leukemic spleen	dCTP ATP	ATP	GTP dGTP	dTTP

Table III Ribonucleotide reductase activity of peripheral leukemic leukocytes [15, 17]

Diagnosis	Number of patients	dCDP, $\mu\text{mol mg}^{-1}\text{h}^{-1}$	
		Crude extract	Eluate through sephadex G 20
AL	4	$70 \pm 60^*$	$160 \pm 100^*$
CML	9	78 ± 12	297 ± 16.2
CLL	3	13 ± 13	20 ± 20
Normal	4	03 ± 02	0 ± 0

* Mean \pm standard errorTable IV The effect of 5-deoxyadenosyl B₁₂ on ribonucleotide reductase activity of human megakaryoblastic bone marrow [16]

Enzyme source 5-deoxyadenosyl B ₁₂ concentration (M)	Substrate	dCDP formed, pmol/mg protein/h				
		0	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Bone marrow	CTP	14	56	70	78	72
<i>L. leichmannii</i>	CTP	29	1 679	1,903	2 063	2,051
<i>L. leichmannii</i> plus bone marrow	CTP		1 462	1,974	1,904	
Bone marrow	CTP	14	12	10	9	

Table V Comparison of ribonucleotide reductases

Parameter	<i>E. coli</i>	<i>L. leichmannii</i>	Rat Novikoff hepatoma	Mouse leukemic spleen	Human leukemic leukocyte
Level of phosphorylation substrate	Diphosphate	Triphosphate	Diphosphate	Diphosphate	Diphosphate
Requirement for cobamide coenzyme	no	yes	no	no	no
Physiological hydrogen donor	Thioredoxin	Thioredoxin	Enzyme S		
Allosteric regulation of substrate specificity	yes	yes	yes	yes	yes
dATP as general negative effector	yes	no	yes	no	
Requirement for Mg^{++}	absolute	relative	absolute	relative	

is that dCTP stimulates CDP reduction and GTP stimulates ADP reduction in mouse leukemic spleen. However, no marked differences were found between the enzymes purified from leukemic spleens, normal spleens, and spleens of control animals injected with phenylhydrazine. The activities were too low to allow any conclusion concerning inhibition by negative effectors.

Ribonucleotide reductase is present in human leukocytes from blood of patients with acute or chronic myelocytic leukemia. Much lower specific activities were demonstrated in lymphocytes from patients with chronic lymphocytic leukemia and even lower levels in normal leukocytes. The levels of ribonucleotide reductase activities in the crude extracts and the enzymes, which were subjected to gel filtration on Sephadex G-25 to eliminate endogenous nucleotides, are shown in table III. Leukocytes from patients with acute leukemia or chronic myelocytic leukemia retain a proliferative capacity which has largely disappeared from the cells found in normal blood and patients with chronic lymphocytic leukemia. The properties of the enzyme of leukocytes from chronic myelocytic leukemia were investigated. There is an absolute requirement for a hydrogen donor. Ribonucleoside diphosphates are the preferred substrates. CDP reduction was stimulated by the presence

of ATP [17]. Further investigations is needed to establish the mode of allosteric regulation of human ribonucleotide reductase.

The ribonucleotide reduction by ribonucleotide reductase from *L. leichmannii* requires coenzyme-B₁₂ [18] and the reduction occurs at the triphosphate level [19]. It is important to confirm whether coenzyme-B₁₂ is involved in human bone marrow system. As shown in table IV, the ribonucleotide reductase from the B₁₂ deficient human bone marrow was not stimulated by the addition of several levels of coenzyme B₁₂ [16]. The reduction by *L. leichmannii* enzyme and the mixed enzyme were greatly stimulated by coenzyme B₁₂.

Table V shows a comparison of the characteristics of ribonucleotide reductase from different sources. Ribonucleotide reductase of animal system, including human leukocytes, resembles that of *E. coli* in its substrate specificity, non requirement of coenzyme B₁₂, and the influence of nucleotide effectors. However, some differences exist in the mode of the effectors influences.

Ribonucleotide reductase is the enzyme which is required for deoxyribonucleotide synthesis. The allosteric control of deoxyribonucleotide synthesis by ribonucleotide reductase may play one of the regulatory roles in DNA replication in leukemic leukocytes. Studies on ribonucleotide reductase is important for chemotherapy on leukemia because hydroxyurea inhibits the human ribonucleotide reductase action [16].

Acknowledgement I wish to thank Dr. ROBERT SILBER, New York University, for his support and collaboration in this investigation.

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Repeated Sequences in Human Leukemic DNA

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Abstract The DNA extracted in macromolecular form from human leukemic leucocytes has been fractionated in $\text{Ag}^+ \cdot \text{Cs}_2\text{SO}_4$ and $\text{Hg}^{++} \cdot \text{Cs}_2\text{SO}_4$ preparative density gradients. Three satellite DNAs and a repeated DNA fraction have been isolated and their properties have been compared in leukemic and normal tissues. So far, no difference has been found with the techniques used.

Key Words

Leukemic cells

DNA structure

Ultracentrifugation of DNA

Human genome contains the following different groups of families of nucleotide sequences having different complexities, that is, different repetition frequencies: (1) highly repeated sequences, corresponding to the nuclear satellite DNAs, which form distinct bands in equilibrium density gradients in the analytical ultracentrifuge, (2) the so-called intermediate fraction which has an intermediate repetition frequency, (3) the heterogeneous not repeated DNA which accounts for approximately 80% of the total genome [1-2]. This has been established by studies on the kinetics of renaturation of the different DNA fractions [3].

These different DNAs have been isolated from the bulk of human DNA, extracted in macromolecular form from human leukemic leucocytes, by silver- and mercury-caesium sulfate centrifugation and some of their properties have been studied.

Materials and Methods

Ultracentrifugation of DNA in $\text{Ag}^+ \cdot \text{Cs}_2\text{SO}_4$ density gradients The DNA was extracted from human leukemic leucocytes as previously described [4]. Analytical ultracentrifuga-

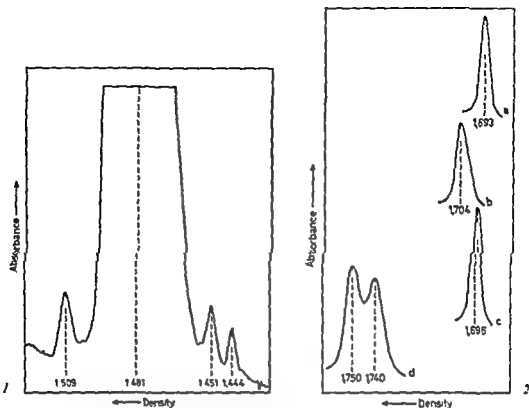


Fig 1 Human leukemic DNA centrifuged to the equilibrium in an $\text{Ag}^+\text{-Cs}_2\text{SO}_4$ density gradient in the analytical ultracentrifuge

Fig 2 Isolated human satellite DNA II centrifuged to equilibrium in neutral CsCl in native a, denatured b and renatured c conditions and in alkaline CsCl d

tion of human leukemic DNA in $\text{Ag}^+\text{-Cs}_2\text{SO}_4$ density gradients and the isolation of satellite DNAs from $\text{Ag}^+\text{-Cs}_2\text{SO}_4$ preparative gradients were carried out according to the techniques reported in a previous paper [2]

Ultracentrifugation of DNA in $\text{Hg}^{++}\text{-Cs}_2\text{SO}_4$ density gradients The technique reported by CORNEO *et al* [2] was used

Ultracentrifugation of DNA in neutral and alkaline CsCl gradients, and denaturation and renaturation of DNA This was carried out according to techniques currently used in our laboratory [2, 5, 6]

Results

Different DNA fractions, binding different amounts of silver ions, change their densities to a different extent, so that they band at different levels in a density gradient. Figure 1 shows a densitometer tracing of human leukemic

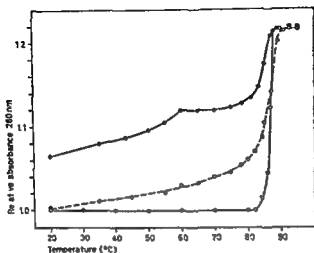


Fig 3 Absorbance temperature curves of isolated human satellite DNA II in native (○—○—○) denatured (● ● ●) and renatured (○—○—○) conditions.

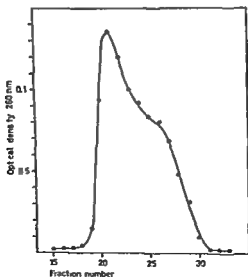


Fig 4 Fractionation of total human DNA in a preparative Hg Cs_2SO_4 gradient

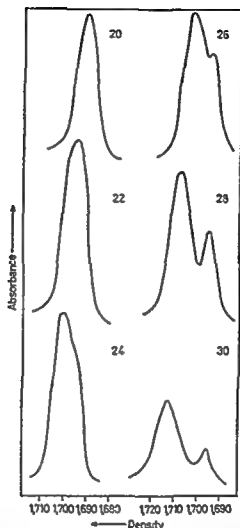


Fig 5 Patterns in neutral CsCl of different DNA fractions obtained from preparative $\text{Hg}^{++}\text{-Cs}_2\text{SO}_4$ centrifugation. The numeration of the fractions corresponds to that of figure 4.

DNA centrifuged to the equilibrium in $\text{Ag}^+\text{-Cs}_2\text{SO}_4$ in the analytical ultracentrifuge. The main peak is the main heterogeneous not repeated DNA, which in this experiment comprehends also the intermediate fraction. The lightest peak is satellite DNA I, which has a density of 1.687 g/ml in neutral CsCl [7]. The peak on the heavy side is satellite DNA II, which has a density of 1.693 g/ml in neutral CsCl [2], and the peak between the main peak and the lightest peak is a new satellite, called satellite DNA III, which has a

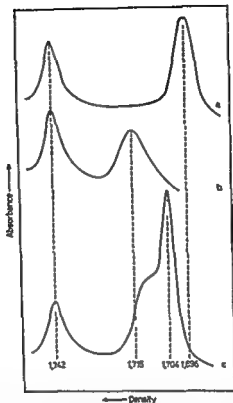


Fig 6 Human intermediate repeated DNA, obtained as described in the text, in native *a*, denatured *b* and renatured *c* conditions, centrifuged to equilibrium in neutral CsCl in the analytical ultracentrifuge. The peak on the left in each tracing corresponds to the density marker, 2c phage DNA.

density of 1.696 g/ml in neutral CsCl, and whose properties are now under study.

In figure 2 some properties of satellite DNA II are analyzed. All satellite DNAs, although differing one from the other, have general properties in common. The first tracing (fig 2a) shows the native density in neutral CsCl; after heat denaturation the density increases as for any double-stranded DNA (fig 2b), and after renaturation the density tends to go back to the native value (fig 2c). If the satellite DNA is centrifuged in alkaline CsCl the

2 complementary strands separate having a bias in the distribution of purines and pyrimidines (fig 2d)

Figure 3 shows the absorbance temperature curve of satellite DNA II, in native (fig 3a), denatured (fig 3b) and renatured (fig 3c) conditions. From the curve after renaturation it appears that this DNA has in such condition partially regained its native double stranded structure. This is an index of the low complexity of satellite DNA compared to the main heterogeneous DNA.

Figure 4 shows the pattern of human total DNA in preparative mercury-caesium sulfate centrifugation. In the shoulder, which is evident on the light side of the peak, a DNA fraction which displays the properties of the intermediate fraction [1] is located.

Figure 5 shows the patterns in CsCl of the fractions corresponding to the shoulder region of the gradient of figure 4, after extensive dialysis to get rid of the mercury ions. The smaller peak in each fraction of this slide is the intermediate DNA. This has been isolated by a subsequent preparative centrifugation in neutral CsCl, and, as shown in figure 6, it has then been analyzed in the analytical ultracentrifuge in neutral CsCl in native (fig 6a), denatured (fig 6b) and renatured (fig 6c) conditions. Also this DNA tends in part to renature.

The replication of satellite DNAs has been studied by incubating *in vitro* human leukemic bone marrow with tritiated thymidine. This was incorporated to the same extent by the main DNA and the satellite DNAs. More interesting will be to carry out this experiment in synchronized human cells to establish whether satellite DNAs are replicated in a specific time during the S period.

Discussion

This investigation has been carried out on DNA extracted from human leukemic leucocytes. So far no definite difference has been found between leukemic DNA and human normal DNA obtained from human placenta [2] by the techniques used. Other techniques, like hybridization between normal and leukemic DNA fractions, or the direct analysis of nucleotide sequences, like the investigation of the pyrimidine tracts [8] will be used.

Further investigations will be carried out on the localization of satellite DNAs in the cell. There is evidence that in mammalian species, satellite DNAs are located in the constitutive heterochromatin [9]. Furthermore by *in situ* hybridization experiments mouse satellite DNA appears to hybridize specifically with the centromeres of chromosomes during mitosis [10]. The

relevance of these recent experiments in relation with a possible function of repeated DNAs in the mitotic cycle is obvious

Acknowledgments This work was supported by a grant from the Consiglio Nazionale delle Ricerche (Rome)

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actions takes place, in which 45S precursors are converted to smaller intermediates (41S and 36S), and finally to 32S RNA which is subsequently cleaved to yield 28 and 18S RNA. Some of the RNA is degraded in the process. If one looks at the overall balance sheet, about 50% of the 45S ribosomal RNA precursor is made up of sequences that are ultimately used to make ribosomes. The other half of the molecule is synthesized and then apparently destroyed.

It is well known that ribosomal RNA functions in the cell in close association with protein packaged as ribosomal units. A single ribosome consists of subunits containing 28S RNA and 18S RNA. It appears likely that the first association of ribosomal RNA with protein to form the subunits of these ribosomes occurs within the cell nucleus. Thus, 28S RNA is packaged to form a 60S subunit, and 18S RNA is packaged with protein to form a 40S subunit. These subunits rapidly leave the nucleus for the cytoplasm to form 80S single ribosomes which are soon utilized for protein synthesis.

At this point, one must mention for the sake of completeness the 5S RNA moiety, this is contained within all the mature ribosomal RNA particles which contain 28S RNA. It is also contained in the nucleolar particles which contain ribosomal precursor RNA. It is clear that although this small 5S RNA is associated with the larger 45S RNA precursor, it is not a part of the larger molecule. The precise place of 5S RNA in the cell economy is at the moment uncertain.

Let us now turn our attention to other cellular RNA species. The role of transfer RNA in protein synthesis and the chemistry of this molecule are far better known than those of any other RNA species. However, very little is known about the site of transfer RNA synthesis in the cell.

While the ribosome and transfer RNA molecules have roles to play in the synthesis of every protein within the cell, the actual dictation of amino acid sequence is accomplished by the messenger RNA. Many events in animal cells are expressions of the changing patterns of protein synthesis, for example, differentiation and cell division. These processes must revolve around the availability of messenger RNA.

In bacteria, messenger RNA represents a small proportion of the total cellular RNA and is rapidly synthesized and degraded. Therefore, if bacterial cells are exposed to labeled precursors for only a brief period of time, then the amount of radioactivity in messenger RNA relative to that in other RNA species is high enough so that messenger RNA can be studied. Another point of importance in bacteria is that ribosomal RNA represents a distinct species of RNA and does not therefore necessarily reflect the average composition of the total cellular DNA. On the other hand, messenger RNA in bacteria comes from many genes and is presumed to reflect the average DNA composition, therefore, it is a DNA-like RNA.

In animal cells, if cytoplasmic extracts of briefly labeled cells are examined, it is found that about 5 to 10% of the total incorporated radioactivity is cytoplasmic, rapidly labeled RNA. In the polyribosomes of cells labeled with ^3H uridine for 30 min or less, labeled RNA can be recovered free from protein. Two features of this polysome-associated, rapidly labeled RNA will be recognized: (1) it sediments more slowly than the heterogeneous nuclear RNA of high molecular weight, (2) it does not conform to the sedimentation pattern of either ribosomal or transfer RNA. In addition, it has been found that the base composition of this polysomal RNA is very different from the other RNA species and is more similar to cellular DNA. This rapidly labeled, DNA-like RNA species from polysomes has been considered to represent mammalian messenger RNA.

To summarize the major features of *RNA synthesis in mammalian cells* Under the influence of RNA polymerases, ribonucleotide triphosphates are polymerized into a chain which is a transcription of one of the twin strands of DNA. It is likely that only certain regions of the chromosomal DNA are transcribed, those corresponding to the euchromatin. The nucleolus appears to be a principal site of ribosomal RNA synthesis before its transfer to the cytoplasm to form the cytoplasmic ribosomes. The principal species of RNA made by the mammalian cell are a low molecular weight 4S RNA which is principally transfer RNA, a high molecular weight RNA which undergoes rearrangement to yield 28 and 18S ribosomal RNA moieties, and messenger RNA which is tentatively identified as heterogeneous and of relatively low molecular weight. In addition, considerable quantities of 5S and several other RNA species are synthesized, the function of these 'minor' RNA components is not known, but all are found in the RNA fraction which 'reads' the genetic code. Kinetic evidence suggests that there is a very high turnover rate of nuclear RNA, a considerable fraction of which is short lived and never reaches the cytoplasm.

It appears that these basic features of RNA metabolism are also true for human leukocytes, although we are still uncertain about the sites within these cells in which different events in RNA metabolism take place.

What are the interesting or unusual features of *RNA metabolism in normal and malignant human leukocytes*? Let us consider first the rate of RNA synthesis and of RNA turnover in human leukocytes. It was apparent to the hematologists of the ninety forties and fifties that leukemic blast cells had more RNA than did differentiated end cells. By the early nineteen-sixties it was established that the lymphoblast and the myeloblast also had very high rates of RNA synthesis and turnover. This involved all the classes of RNA then defined: ribosomal RNA, 4S RNA, and heterogeneous, rapidly labeled RNA. An unexpected and interesting observation was that the abnormal leukocytes of patients with infectious mononucleosis had extremely high RNA turnover rates, equivalent to those of blast cells. Another unexpected observation was that granulocytes had significant levels of RNA synthesis despite the absence of a well developed endoplasmic reticulum and identifiable nucleolus.

What are the factors influencing RNA synthesis and turnover in human leukocytes? To answer this question, one must consider the individual types of white cells. Let us first look at the lymphocyte and the plasma cell. In cells derived from lymph nodes of hyperimmunized animals, protein synthesis occurs on ribonuclease sensitive polyribosomes. The size of poly-

ribosomal aggregates increases during the course of immunization. A polyribosomal population containing protein with the antigenic determinants of γ -globulin was found by NORTON to be composed of aggregates of 5 to 10 ribosomes. He considered the size to be consistent with a messenger RNA molecule in lymphoid cells large enough to code for polypeptides in the range of 20,000 to 40,000 molec wt. Additional studies indicated that the heavy chain is synthesized as a single polypeptide unit, rather than assembled from a number of smaller chains.

An important observation in myeloma cells was that heavy chains and light chains appeared to be made on different classes of polyribosomes. Certain plasma cell tumors of mice and men appear to synthesize an excess of the light chain of γ -globulin. This may be a malignant counterpart of a normal process, since lymph node cells from normal immunized animals also produce an excess of light chains relative to heavy chains.

RNA synthesis in lymphoid cells can be influenced by external factors. It is now well established that enhanced RNA synthesis precedes the increased protein synthesis and thymidine incorporation of peripheral blood and lymph node lymphocytes exposed to specific antigens or to nonspecific mitogens such as phytohemagglutinin. The increased synthesis induced by specific antigens and phytohemagglutinin involves all classes of phenol-extractable RNA that can be differentiated.

The response of lymphocytes to phytohemagglutinin is reduced in patients with chronic lymphocytic leukemia, certain lymphomas, and in patients receiving chemotherapy. It is also reduced by the addition of the antiviral agent amantadine, by corticosteroids, and chloroquine.

Interestingly there is no convincing evidence at present that stimulating agents such as specific antigen and nonspecific mitogen increase the production of γ -globulin or of specific antibody. Therefore, the reason for the enhanced RNA turnover is uncertain, although we do know that blocking of RNA synthesis will also block the blastogenic response of lymphoid cells to specific antigen and to phytohemagglutinin.

What factors modify the RNA metabolism of the differentiated granulocyte? The best defined is phagocytosis. After particle ingestion there is an increase in the rate of entry into the cell of labeled RNA precursors and the specific activity of the acid soluble nucleotide pool of the granulocyte increases. Pre-existing granulocyte RNA is broken down at an accelerated rate and new RNA is formed. In other words, with phagocytosis the RNA turnover rate is increased. What role does this increased turnover rate serve? The answer is not known. Although the changes in RNA metabolism with

phagocytosis are among the early post phagocytic events, blocking of RNA synthesis has no effect on the other metabolic changes accompanying particle ingestion. Thus, the post phagocytic degranulation and increase in glucose oxidation and lactate production appear to be triggered by particle ingestion itself, and do not require RNA or protein synthesis. An interesting recent observation is that the production of leukocyte pyrogen following phagocytosis does require new RNA synthesis.

Messenger RNA There have been no direct measurements of the stability or turnover rate of messenger RNA in mammalian leukocytes. All measurements have been indirect and based on the rate of decay of protein synthesis after inhibition of RNA synthesis with actinomycin D. Such measurements have shown a fall in the rate of protein synthesis, the half life being between 30 and 60 min in populations of granulocytes and myeloblasts, a rate which is slower than that of the messenger RNA turnover of bacteria but faster than that of liver cells and immature red cells. Interestingly, the highest turnover rate of messenger RNA thus far recorded in man was in a patient with macroglobulinemia and lymphosarcoma with bone marrow and blood stream invasion. The circulating malignant lymphoid cells were manufacturing IgM. The half life of messenger RNA synthesis in these cells, measured indirectly, was about 30 min.

We now come to the last area of current interest concerning the RNA metabolism of human leukocytes. The interaction of mononuclear phagocytes with lymphocytes in certain phases of the immunologic response is supported by several observations. (1) Pure populations of lymphocytes containing no phagocytes show a reduced blastogenic response to specific antigen *in vitro*. Addition of phagocytes restores response to antigen.¹ (2) Human monocytes and macrophages exposed to antigen *in vivo* and washed free of extracellular antigen can induce transformation of autologous lymphocytes; together they form an immunologic island.² (3) Normal non-immune lymphoid cells can induce antibody synthesis in irradiated recipient animals, if living macrophages containing antigen are also transferred.³ (4) Close contact and cytoplasmic connections have been demonstrated between lymphocytes and macrophages *in vitro*.⁴ (5) RNA prepared from animal macrophages exposed to antigen can induce the corresponding

response to produce specific antibody. This was called the

instruction hypothesis Several recent studies make the RNA instruction hypothesis unlikely. It is clear, however, that macrophage RNA containing minute amounts of antigen can indeed induce specific antibody synthesis by lymphocytes. Furthermore, it is clear that the macrophage RNA enhances the immunogenicity of these minute amounts of antigen. In other words, macrophage RNA may act as an adjuvant.

It is clear that these studies of RNA synthesis by normal and abnormal human leukocytes should now be repeated in the light of the more sophisticated knowledge of RNA metabolism in mammalian cells in general and with the more refined techniques now available, such as DNA-RNA hybridization and polyacrylamide gel electrophoresis. For example, these obvious questions arise: (1) Is the pattern of RNA synthesis and control different in the normal and the malignant cell? (2) In the cells of infectious mononucleosis, are there RNA species homologous to the DNA of Epstein-Barr virus? (3) Are there multiple forms of RNA polymerase controlling cell differentiation in the leukocyte as in the sea urchin and the hepatic cell? (4) What is the influence of viral transformation? The answers to these questions are contained in areas of investigation which are both fruitful and demanding.

RNA Metabolism of Circulating Lymphocytes Studied in Man After Autotransfusion and *in vitro* ^3H -Cytidine Labeling¹

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Abstract The labeling pattern of *in vitro* ^3H -cytidine labeled blood lymphocytes was studied after autotransfusion in 2 hematologically normal patients and in 6 patients with untreated chronic lymphocytic leukemia. An *in vitro* incubation time of 45 min results in a rapid loss of label during the first hours, whereas after a 4 h incubation time the label is much more stable. The results are taken to indicate that during a 45 min incubation time predominantly metabolically unstable RNA fractions become labeled while after a 4 h *in vitro* incubation period a larger proportion of the stable RNA fractions is labeled.

Key Words

Autoradiography
Chronic lymphocytic leukemia
Lymphocytes
RNA metabolism

There are only a few data concerning the *in vivo* RNA metabolism of cells in human beings [13, 20, 22]. Thus we have designed experiments to study the RNA metabolism of circulating human blood lymphocytes in patients with normal hemopoiesis and with untreated chronic lymphocytic leukemia (CLL). This has been achieved by the method of autotransfusion of *in vitro* ^3H cytidine labeled blood lymphocytes [9-11]. In earlier studies it was demonstrated that practically all lymphocytes of hematologically normal persons and of patients with CLL are actively engaged in RNA synthesis but not in DNA synthesis [2-4]. The *in vitro* labeling procedure with ^3H -cytidine results in the labeling of close to 100% of all human blood lymphocytes. ^3H -cytidine was incorporated into lymphocytes far more extensively than the RNA specific labeling substance ^3H -uridine [4]. ^3H cyti-

¹ Research supported by Euratom contracts No 031-64-1 BIAD and No 072 68 1 BIOD and the Bundesministerium für Wissenschaftliche Forschung

dine is not only a suitable label for human blood lymphocytes but also – due to the very low number of cells in DNA-synthesis – may be regarded under these circumstances to be a specific RNA marker. The basic studies on the labeling of lymphocytes and their *in vitro* RNA turnover pattern have been published elsewhere [3, 4].

During the *in vitro* incubation of the lymphocytes with ^3H -cytidine newly synthesized RNA will become labeled. Further ^3H -cytidine incorporation will be stopped by the addition of a large amount of unlabeled cytidine and by the subsequent rapid autotransfusion of these labeled lymphocytes. The pattern of the loss of the labeling intensity studied in autoradiograms of the autotransfused, circulating lymphocytes will reflect their *in vivo* RNA metabolism.

Materials and Methods

The autotransfusion studies have been performed in 2 hematologically normal persons and in 6 patients with an untreated chronic lymphocytic leukemia. The peripheral blood lymphocyte count varied among the 6 CLL-patients between 10 000 and 500 000 mm^3 blood.

One unit of venous blood (500–600 ml) was collected in a plastic bag containing 75 ml of ACD (Biostat S-rum Institut Frankfurt/Main, FRG, ACD Stabilisator USP XVII A) and was incubated with 500–2,000 μCi of ^3H -cytidine (spec. Act. 4.5 Ci/mM (Schwarz Bio-research Inc., Orangeburg, N.Y., USA) or 2.0 Ci/mM (Radiochemical Center, Amersham, England) for 45 min or 4 h at 20 or 37°C. At the end of the incubation period non-radioactive cytidine was added to the bag in a concentration 1 000 times the quantity of cytidine in the radioactive material. Thereafter the blood unit was autotransfused into the patients within 10–20 min.

After completion of autotransfusion 2–4 ml of venous blood were removed at serial time intervals. 10–15 samples were taken during the first day, 4 on the following day, 3 on day 3 to 5, and 2 daily until day 7 or 10 and then once a day for the following week. Thereafter samples were taken at greater intervals. The blood was mixed immediately with 1 ml of a EDTA – plasmagel mixture in order to prepare leukocyte concentrates after erythrocyte sedimentation [3, 4]. The cell concentrate smears were immediately air dried, fixed in methyl alcohol (3×10 min) and processed for autoradiography.

A modified stripping film technique of autoradiography was used according to the method described originally by PETC [4, 17]. The exposure times of the autoradiographs ranged from 42 to 310 days and were determined on the basis of the intensity of labeling in control slides.

At the time intervals studied at least 2,000 small lymphocytes were evaluated for their labeling intensity in the autoradiographic smears. The mean grain count determination was based on a minimum of 50 to 100 labeled lymphocytes per slide.

Because the background varied in the slides from patient to patient, an arbitrary background limit was chosen for each patient. In the leukocyte concentrate smears of patient E.F. and M.S. all lymphocytes with 4 grains or more were considered to be labeled.

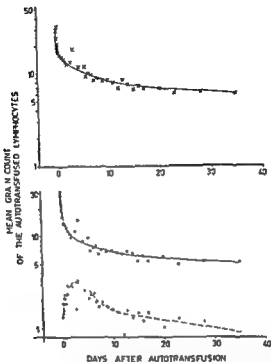


Fig 1 Changes in the labeling intensity of the autotransfused circulating lymphocytes of a patient (A G) with CLL after a 45-min *in vitro* incubation time with ^3H -cytidine. The upper part of the diagram gives the changes in the total cell labeling intensity (x—x) in the lower part the changes of the nuclear (●—●) and cytoplasmic (---C) label are shown separately.

A background limit of 5 grains was chosen for the patients V P, F S, M R, and A G and of 7 grains in patients M K and A G.

Results

Figure 1 shows the decrease of the labeling intensity of the autotransfused, labeled lymphocytes in a patient with CLL after a short *in vitro* incubation time of 45 min. Whereas the percentage of labeled lymphocytes circulating in the blood did not change significantly during the first hours after autotransfusion [10, 11], the total cell labeling intensity shows during this

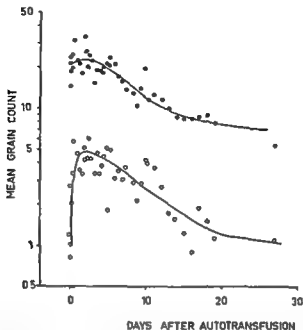
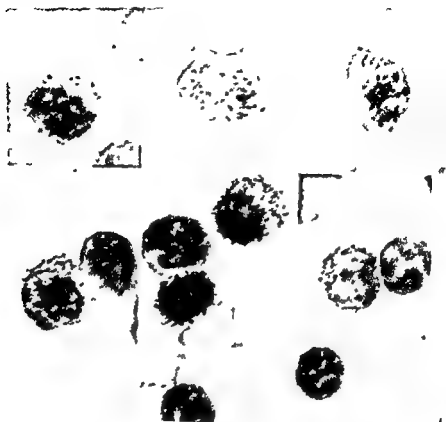


Fig 2 Changes in the labeling intensity of the autotransfused circulating lymphocytes of a patient (M S) with CLL after a 4 hours *in vitro* incubation time with ^3H cytidine, given for the total cell labeling (●—●) and the cytoplasmic label (○—○) separately

time a rapid loss in labeling intensity of about 50% (upper part of the diagram). In the following hours and days this rate of diminution of labeling intensity slows down and after about 10 days the mean grain count reaches a relatively stable phase.

The lower part of figure 1 demonstrates the behavior of the labeling intensity for the cell nuclei or for the cytoplasm separately. Most of the label is seen over the cell nuclei and determines largely the pattern found for total cell labeling. However, there is a distinct increase of labeling intensity over the cytoplasm during the first 2 days after autotransfusion, followed by a slow decline which is nearly parallel to the pattern of the nuclear labeling. This patient is one example out of 3 CLL-patients studied in the same way. In these patients the patterns of the loss of the labeling intensity are similar to these curves (fig 4).

If the usual incubation period of 45 min is prolonged to 4 h, then the labeling intensity of the autotransfused CLL-lymphocytes remains practically constant for the first 5 days and declines slowly thereafter (fig 2). The cytoplasm shows again a marked increase in labeling intensity during the



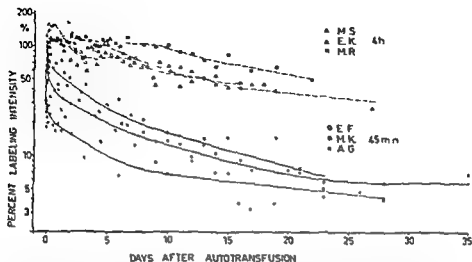


Fig 4 Different rates of loss of the labeling intensity over the autotransfused circulating lymphocytes in 6 CLL-patients demonstrating the different labeling stability after a short (45 min) or a prolonged (4 h) *in vitro* incubation time with ^3H -cytidine. The mean grain count value immediately before autotransfusion is taken as 100% and all subsequent labeling values are expressed as percent of this labeling level.

Figure 4 demonstrates the differences in the loss of the labeling intensity in 6 CLL-patients, in which the lymphocyte incubation time was 45 min in 3 and 4 h in the remaining 3. The mean grain count over the blood lymphocytes of each patient immediately before autotransfusion was taken as 100% and all subsequent values for the labeling intensity in each patient after autotransfusion were expressed as a fraction of the mean grain count before autotransfusion. As indicated above a short, 45-min incubation time results in a marked initial loss of labeling intensity. In contrast, when lymphocytes were autotransfused after a 4-hours incubation time, then the label was much more stable.

The behavior of the labeling intensity according to different *in vitro* incubation times with ^3H -cytidine was investigated not only in leukemic but also in normal lymphocytes after their autotransfusion. The first few results of these studies are shown in figure 5. After the autotransfusion of the lymphocytes incubated only for 45 min with ^3H -cytidine, there seems to be an initial rapid loss of the labeling intensity, followed by an increase with its maximum on the second day. After 10 days, a more stable pattern evolves. The cytoplasmic label increases during the first 3 days followed by a decline nearly parallel to the total cell labeling. The lower part of this dia-

viability during the *in vitro* incubation time. This has been studied previously by comparing the RNA synthesis rates of blood lymphocytes immediately after blood withdrawal with the rates of those lymphocytes which were kept *in vitro* for a time interval of 45 min or 4 h [3, 4]. Comparing the increase of labeling intensity parallel to the duration of *in vitro* incubation time with ^3H -cytidine all these lymphocytes studied showed no distinct difference in their incorporation rates of ^3H -cytidine thereby presumably reflecting unaltered rates of RNA synthesis.

The changes in the labeling intensity of the autotransfused lymphocytes studied are interpreted to indicate their *in vivo* RNA turnover since almost all lymphocytes lost their label after treatment of the cell concentrate smears with perchloric acid or with the specific RNA degrading enzyme ribonuclease [3, 4]. Only about 0.1% or less of the labeled cells remained still heavily labeled demonstrating that these cells were in DNA-synthesis during the time of incubation with ^3H -cytidine.

Because under the conditions of our studies ^3H -cytidine can be considered as a nearly RNA specific labeling substance, the differences in the rates of loss of label according to different long *in vitro* incubation times of the blood unit with ^3H -cytidine should reflect labeled RNA fractions of different metabolic stability. Therefore in the lymphocytes incubated for a short time, 45 min, predominantly unstable RNA fractions appear to become labeled, to which one would have to assign turnover times in the order of some minutes and hours (fig. 1). If the *in vitro* incubation time was prolonged to 4 h, then the autotransfused lymphocytes showed a much more stable labeling pattern. This is taken to demonstrate that in these lymphocytes a larger fraction of more stable RNA with turnover times in the order of days and weeks had become labeled. Similar observations have been obtained in biochemical studies investigating the *in vitro* RNA metabolism of mammalian cells [5, 6, 18, 21]. But it remains to be determined, whether or not the various rates of decline of the labeling intensity observed over the autotransfused lymphocytes can be correlated to the RNA fractions of known different metabolic stability and with well known biochemical characteristics. Appropriate studies are underway.

The fact, that the lymphocytes incubated for 4 h show a much more stable lymphocyte label than those incubated only for 45 min, may not only be explained by a larger fraction of metabolically more stable RNA labeled during a 4-hours incubation time. In addition there may be a larger pool of volatile, intracellular tritiated compounds from which intracellular reutilization may occur. There is also a large amount of tritiated RNA break-

down products from the labeled metabolically unstable and therefore rapidly decaying RNA fractions which then may be incorporated into the more stable RNA molecules. Furthermore there is an intracellular acid soluble pool which may contain a larger quantity of labeled RNA precursors and which may then feed into the continuous RNA-synthesis resulting in a more stable labeling of these lymphocytes. This acid soluble RNA precursor pool is not detected in the present studies by autoradiography because it is extracted by methyl alcohol fixation [1]. The addition of an excess amount of non-radioactive cytidine to dilute the radioactivity in the acid soluble RNA pool and later of the intracellular located RNA breakdown products apparently did not completely prevent the intracellular reutilization of this tritiated material [7, 8].

In this study a wave of tritium labeling was found over the cytoplasm indicative of the movement from the nucleus to the cytoplasm of tritium labeled material, which is presumably RNA. That RNA moves from the cell nucleus into the surrounding cytoplasm was demonstrated also in many other investigations [12, 15, 16, 18, 19, 23].

The labeling patterns of the autotransfused normal lymphocytes showed a more complex curve with some decrease and increase in the labeling intensity. These undulations have yet to be analyzed in more detail and in a larger number of patients. If they are reproducible, then they could reflect waves of recirculating, intensively labeled lymphocyte populations. Preliminary results have shown that the mean labeling intensity of the recirculating lymphocytes in the thoracic duct lymph is higher than in the blood lymphocyte population (unpublished data).

Whether or not the *in vivo* RNA-metabolism of leukemic and normal lymphocytes is different from each other, remains to be determined.

Acknowledgements These studies would not have been possible without the continuous support of the members of the Department of Hematology, University of Ulm, and its director Prof. Dr. H. HEIMPEL. The valuable technical assistance of Mrs. M. BREUER and Miss L. RABUS is gratefully acknowledged.

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Autoradiographic Studies on the Kinetics of Nuclear-Cytoplasmic Transfer of RNA in Blast Cells of Acute Leukaemia

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Abstract Autoradiographic investigations on normal PHA-stimulated lymphocytes and on blast cells of acute leukaemia were carried out in order to evaluate the nuclear-cytoplasmic shift of ^3H -uridine and the degree of nucleolar labelling with the same radioactive precursor. The results obtained indicate that, in contrast with PHA blast cells, a variable proportion of leukaemic blasts, even after prolonged periods of incubation, show a delayed transfer of uridine to the cytoplasm, as well as a reduced concentration of label in the nucleoli. The implications of these findings are discussed also in the light of recent biochemical acquisitions.

Key Words

Autoradiography
Leukemic cells
PHA stimulation
Transfer of RNA

When normal human lymphocytes, stimulated by phytohaemagglutinin (PHA), are incubated *in vitro* with an RNA radioactive precursor, such as uridine-5-T, labelling occurs at first over the nucleus and subsequently, after the first half-hour, also in the cytoplasm [3]. When performing chase studies on PHA-induced blasts, it may be observed that nuclear grain counts decrease from the 4th to the 6th hour, while the cytoplasmic counts reach a peak at 5 h [3].

In leukaemic cells, autoradiographic studies with uridine-5-T [5] have confirmed the early work of GAVOSTO *et al* [1], who reported that uptake with labelled RNA precursors was much diminished compared with that of normal immature cells of the haemopoietic system. Chase studies on leukaemic cells have instead given some controversial results. QUAGLINO and HAYHOE [5] suggested that the nuclear-cytoplasmic shift of label might be delayed in a variable proportion of leukaemic cells, while later studies of SINKS and HAYHOE [6] appeared to indicate that the time sequence of label transfer from nucleus and nucleolus to cytoplasm did not differ essentially

from that seen in PHA induced blast cells and in other cultured mammalian cells

We have therefore carried out a further series of autoradiographic investigations in an attempt to clarify this controversial issue. We have also studied as accurately as possible the uptake and distribution of label within the nucleolus, since SIVAS and HAYHOE [6] did not report any variation in nucleolar grain counts in 3 cases of acute leukaemia. It is clearly important to assess the functional significance of nucleoli, since in leukaemic cells these organelles are more developed and prominent than in normal immature cells [2, 4].

Material and Methods

Our cases consisted of 3 normal subjects, whose peripheral blood lymphocytes were incubated with PHA and 9 cases of untreated acute leukaemias: 3 myeloblastic, 2 lymphoblastic and 2 myelo-monocytic.

Samples of cells from each case were cultured in a medium consisting of the patient's serum and medium TC 199, the cell concentration being adjusted to approximately 2,500-3,000 cells/ml.

Uridine 5-T at a concentration of 0.2 and 0.5 μ Ci/ml and tritiated thymidine at a concentration of 0.5 μ Ci/ml were added to different samples from each case studied. Cell samples were obtained at 30 min, 2 and 4 h, and autoradiographic preparations were made.

At least 100 cells from each sample were counted. The results were evaluated separately in the nucleus and in the cytoplasm.

Results

Table I shows the results. Leukaemic cells showed considerably lower total grain counts than normal PHA cells. However the greatest difference was observed in the cytoplasm, leukaemic cells showing in all cases a much lower uridine incorporation. It is noteworthy that even in those cases of acute leukaemia with fairly high nuclear grain counts (cases 2, 3, 7, 8), the shift of label from nucleus to cytoplasm, after 4 h of incubation, was much delayed compared to normal PHA cells. In the latter the percentage of cytoplasmic grains varied from about 30 to 40%, while in leukaemic cells it was usually well below 15% with one exception (fig. 1).

The nuclear grain counts obviously refer to the whole of the nuclear area, but it is necessary to emphasize that while for PHA blasts this means that a

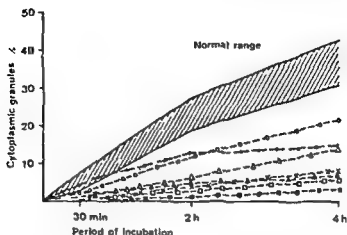


Fig 1 Percentage of cytoplasmic grains in normal PHA blasts and in leukaemic cells after 30 min 2 and 4 h incubation of seven cases

considerable number of granules are concentrated in the nucleolus, in leukaemic blasts the majority of granules are widely scattered over the nucleus and the nucleolus is often poorly labelled or labelled at the periphery (fig 2 and 3). Within a given leukaemic cell population, there is an extreme variability of nuclear/nucleolar labelling and in parallel of nuclear-cytoplasmic shift in different cells (fig 4). In particular it has been possible to observe quite frequently cells whose uptake was many times greater than the average grain counts of the majority of other leukaemic blast cells (fig 5 and 6).

There was a positive correlation between the degree of uridine uptake and the percentage of thymidine incorporation, i.e. the proliferative activity of the cells. The cases with the lowest rates of RNA synthesis showed also a low percentage of thymidine incorporation (table 5).

Actinomycin chase studies, carried out in 3 cases of acute leukaemia, showed a considerable reduction in total grain counts, but a distribution of label not significantly different from that observed in the untreated specimens.

Fig 2 Group of 3 blast cells from a case of acute leukaemia. The lower blast cell shows a prominent nucleolus which is labelled only at the periphery.

Fig 3 Group of leukaemic blasts showing a diffuse nuclear scattering of the label. The largest blast cell also shows a perinucleolar concentration of uridine.



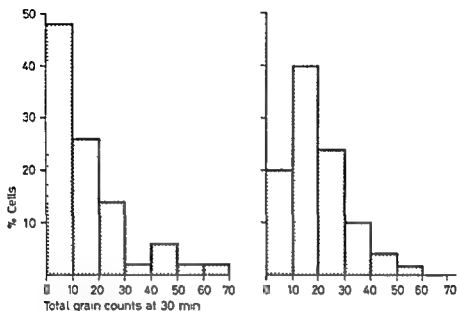


Fig 4 Extreme variability of labelling with uridine in leukaemic cell populations from 2 cases of our series

Discussion

The results concerning the degree of nuclear-cytoplasmic shift of label in acute leukaemia cells seem to confirm the preliminary data reported by one of us a few years ago. The existence of conflicting reports may be explained on the basis of the different approach or evaluation used in each study. For instance, cells with very low grain counts, have been excluded in some studies. This fact may greatly influence the final average grain count and consequently the general pattern.

It is obvious that owing to the low rate of incorporation of the RNA precursor in leukaemic blast cells, the baseline being too high, one will tend to exclude from the final count a significant number of cells, especially after short incubation periods. Consequently the cells taken into account will not reflect the whole cell population, but will comprise mainly that fraction.

Fig 5 Two leukaemic blasts: one of which shows heavy uridine uptake both nuclear and cytoplasmic

Fig 6 Group of 5 blast cells from a case of acute leukaemia. The cell at the bottom left is more heavily labelled than the remaining



Table 1 Average grain counts with uridine 5-T (0.5 µc/ml) in normal PHA and in leukaemic blast cells

Period of incubation	Normal cases			Leukaemic patients								
	1	2	3	1	2	3	4	5	6	7	8	9
				myel	lymph	myel	myel	myel	myel mon	myel	myel mon	lymph
Nuclear												
30 sec	3484	377	361	116	239	144	29	308	222	197	122	57
2 h	412	467	517	149	381	382	1204	1924	275	35	439	143
4 h	7156	824	6614	347	458	544	192	318	3704	426	5912	217
Cytoplasmic												
30 sec	164	280	21	02	198	022	0	0	07	14	003	0
2 h	1426	116	183	04	63	246	018	1	16	52	193	14
4 h	3312	385	4312	24	122	944	092	372	28	79	886	32
* Thymidine uptake	32	34	40	25	12	6	05	09	10	12	8	2

endowed with greater synthetic activity and whose metabolic features, at least from the point of view of the kinetics of RNA transfer from nucleus to cytoplasm, are more akin to those of PHA blast cells

It is worthwhile recalling that in cells such as PHA blasts characterized by intense synthetic activity, this is predominantly (80%) ribosomal, while in cells with a low degree of synthetic activity, as for example circulating lymphocytes [7] and normoblasts C [9] the RNA synthesized appears to be non ribosomal but DNA like and this fraction appears to be metabolized almost exclusively in the nucleus and is not significantly transferred to the cytoplasm. One could therefore suggest that the low average grain counts in a high proportion of leukaemic blast cells is an indication that in these cells the latter type of RNA is predominantly synthesized. This hypothesis would be supported by the fact that the lower the grain count, the lesser the amount of label transferred to the cytoplasm.

However, biochemical studies [8] carried out on some of the cases included in our series, suggest that in leukaemic cells there is an accumulation of unmethylated precursor RNA which is not processed and is not therefore transferred to the cytoplasm.

Our autoradiographic investigations are unable to discriminate between the 2 types of RNA excepting for the fact that the pattern of RNA distribution in leukaemic cells is little affected by actinomycin. Biochemical studies [10] show that following treatment with actinomycin, the largest portion of ^3H uridine still remains associated with nuclear fractions.

Our autoradiographic studies also indicate that a variable, but usually small number of leukaemic blast cells are heavily labelled with uridine-5-T and show a normal nuclear-cytoplasmic shift of label. This observation is in keeping with the fact that in leukaemic cell populations a certain amount of ribosomal RNA is still produced despite the general overall reduction of RNA precursor synthesis [10].

Finally we have observed a positive correlation between uridine uptake and % of thymidine incorporation. If this observation is confirmed, it may be possible to postulate the existence of a direct correlation between the rate of ribosomal formation and the proliferative activity of leukaemic blast cells.

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Impaired Processing of Ribosomal Precursor RNA in Blast Cells of Acute Leukemia¹

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Abstract Homogeneous blast cell populations from 9 cases of acute leukemia were incubated for periods varying from 30 min to 6 h simultaneously with uridine 5-³H and ¹⁴C methyl-methionine. The distribution of label in the different classes of RNA molecules was studied after their separation by sedimentation in sucrose gradients. At variance with normal proliferating blood cells, in leukemic blasts methylation and cleavage of 45 S precursor RNA occurred at a very low rate. Furthermore an accumulation of unmethylated labeled RNA molecules in the size range of ribosomal precursor RNA was observed presumably due to an unbalance between rate of synthesis and rate of processing of 45 S RNA.

Key Words

Leukemic cells

Ribosomes

RNA synthesis

Results obtained in different laboratories suggest that RNA is synthesized in blast cells of acute leukemia at a low rate [3, 9, 11]. This low rate must surely affect ribosomal RNA synthesis since about 80% of total RNA in any type of cell is made up by the latter [2]. However it is hard to reconcile this fact with the existence in acute leukemia cells of highly developed nucleoli [4-8].

The physiology of ribosomal RNA synthesis has been elucidated to a considerable extent in recent years. Nucleolus has been shown to be the site of synthesis of a large 45 S molecule which in the nucleolus itself is then methylated and cleaved through some intermediate size short lived molecules, to a 18 S and a 32 S molecule. The 18 S molecule reaches the cytoplasm immediately the 32 S molecule is converted into a 28 S molecule in the nucleolus before reaching the cytoplasm [5, 6, 10, 16, 17].

In rapidly growing cells such as HeLa cells [5, 16] or PHA stimulated lymphocytes [12] synthesis, methylation and cleavage of the ribosomal

¹ Research supported by a grant from the Consiglio Nazionale delle Ricerche

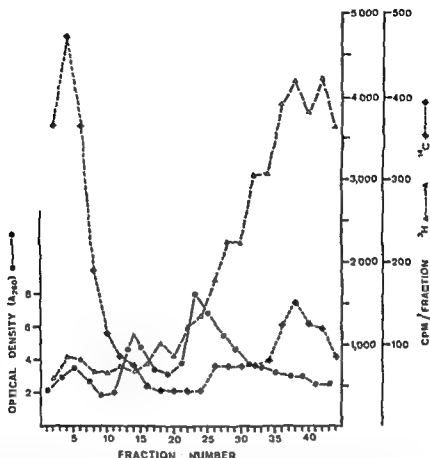


Fig 1 S-dimentation profile of radioactive RNA extracted from leukemic blast cells incubated for 1 h with ^3H 5 uridine ($10 \mu\text{c/ml}$) and ^{14}C methyl methionine ($5 \mu\text{c/ml}$) Centrifugation for 16 h at 16 000 rpm on 5 20% sucrose gradients The absorbance profile is mainly due to unlabeled RNA from AB cells extracted simultaneously

precursor molecule occur very rapidly when the cells are incubated with labeled RNA precursors the label is found associated with 45S RNA after periods as short as 5-15 min while 28S and 18S RNA are labeled after longer periods

The problem of nucleolar function in acute leukemia cells has been tackled in our laboratory by studying in these cells the rate of methylation and processing of ribosomal precursor RNA We have investigated by sedimentation analysis in sucrose gradients the distribution of the label in the various pre ribosomal and ribosomal RNA molecules after different periods of exposure to labeled uridine and methyl labeled methionine Some of our

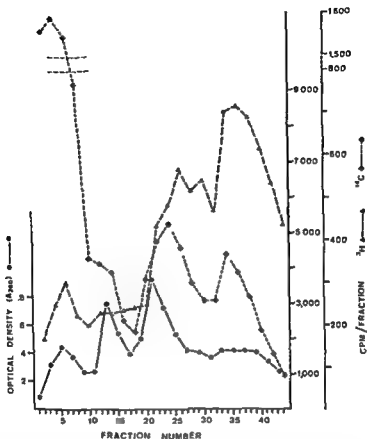


Fig 2 S-dimensionation profile of radioactive RNA extracted from leukemic blast cells incubated for 3 h with ^3H uridine ($10 \mu\text{C}/\text{ml}$) and ^{14}C methyl methionine ($5 \mu\text{C}/\text{ml}$). Centrifugation for 16 h at 16 000 rpm on 5–20% sucrose gradients. The absorbance profile is mainly due to unlabeled RNA from KB cells extracted simultaneously.

results have been already reported [13]. The present paper will give further informations about the data which were obtained in all cases so far examined by the double-labeling technique.

Materials and Methods

The cells studied were obtained from 9 acute leukemia patients chosen because of the homogeneity of their peripheral cell population. Five of them had acute myeloblastic

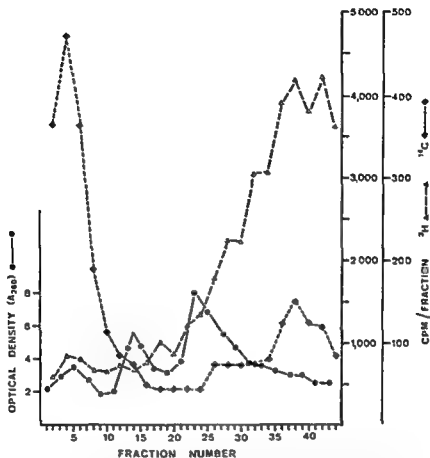


Fig 1 Sedimentation profile of radioactive RNA extracted from leukemic blast cells incubated for 1 h with ^3H 5-uridine (10 μC ml) and ^{14}C -methyl methionine (5 μC ml). Centrifugation for 16 h at 16 000 rpm on 5–20% sucrose gradients. The absorbance profile is mainly due to unlabeled RNA from KB cells extracted simultaneously.

precursor molecule occur very rapidly when the cells are incubated with labeled RNA precursors: the label is found associated with 45S RNA after periods as short as 5–15 min, while 28S and 18S RNA are labeled after longer periods.

The problem of nucleolar function in acute leukemia cells has been tackled in our laboratory by studying in these cells the rate of methylation and processing of ribosomal precursor RNA. We have investigated by sedimentation analysis in sucrose gradients the distribution of the label in the various pre-ribosomal and ribosomal RNA molecules after different periods of exposure to labeled uridine and methyl-labeled methionine. Some of our

radioactivity corresponding to 28S. Furthermore, since formation of 32S RNA was observed in all our cases, simultaneous appearance of a corresponding radioactive 18S peak should have occurred in keeping with the presently accepted scheme of cleavage of 45S RNA [17]. In many of our cases this was not observed, and it is therefore necessary to presume that 18S RNA is for the greatest part degraded soon after its formation.

It must be emphasized however that, in addition to the slow processing of ribosomal precursors, it is apparent from our results an accumulation of unmethylated labeled RNA molecules in the size range of ribosomal precursor RNA. This suggests that circulating leukemic blast cells are characterized by an unbalance between processing of ribosomal precursor RNA and rate of synthesis, thus causing an accumulation of unmethylated precursor. This seems to be a major difference between the metabolic pattern of these cells and that of normal mature cells, such as lymphocytes and normoblasts [12, 14] in which the low rate of ribosomal RNA formation is not associated with any relevant accumulation of unmethylated precursor.

It is interesting to point out that a metabolic pattern of ribosomal precursor RNA similar to that seen in acute leukemia cells may be observed in rapidly growing cells following treatment with protein synthesis inhibitors [1, 15, 18].

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The Application of Cytochemical Methods to the Study of Acute Leukemia

A Review

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Abstract The comparison of cytochemical criteria with conventional morphologic and cytologic criteria as well as with electron microscopic and biochemical investigations all confirm the value of cytochemistry in identifying and classifying acute or immature leukemias. A clearcut and reasonable characterization of the particular leukemic type is useful in improving the quality of treatment.

Key Words

Acute leukemia
Cytochemistry
Esterases in leukemic cells
Leukaemia differentiation
PAS reaction

Cytochemical investigations of the blood and bone marrow cells when based upon the appropriate techniques lead to the demonstration of characteristic patterns in the various types of cells [19-35, 58]. The relationship between these cytochemical staining patterns and certain subcellular structures has been partly elucidated by ultrastructural investigations used in combination with the cytochemical techniques [3, 5, 6, 36, 55] and by cell fractionation and biochemical methods [8, 10, 11, 56]. Thus it may now be generally accepted that acid phosphatase activity is localized in the granules typical of neutrophils, eosinophils and monocytes and in the other less characteristic lysosomal structures of additional blood and bone marrow cells [3, 6, 36, 55]. Alkaline phosphatase [25] seems to be localized in the secondary or specific neutrophil granules which morphologically differ from the primary or azurophil granules containing acid phosphatase [3, 6, 7, 56]. In neutrophils peroxidase [43] is probably localized in the same azurophil granules already mentioned as containing acid phosphatase [3, 6, 10, 11, 56]. In monocytes peroxidase was found to be confined to granules

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The Application of Cytochemical Methods to the Study of Acute Leukemia

A Review

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Abstract The comparison of cytochemical criteria with conventional morphologic and cytologic criteria as well as with electron microscopic and biochemical investigations all confirm the value of cytochemistry in identifying and classifying acute or immature leukemia. A clearcut and reasonable characterization of the particular leukemia type is useful in improving the quality of treatment.

Key Words
Acute leukemia
Cytochemistry
Esterases in leukemia cells
Leukemia differentiation
PAS reaction

Cytochemical investigations of the blood and bone marrow cells when based upon the appropriate techniques lead to the demonstration of characteristic patterns in the various types of cells [19 35 58]. The relationship between these cytochemical staining patterns and certain subcellular structures has been partly elucidated by ultrastructural investigations used in combination with the cytochemical techniques [3 5 6 36 55] and by cell fractionation and biochemical methods [8 10 11 56]. Thus it may now be generally accepted that acid phosphatase activity is localized in the granules typical of neutrophils, eosinophils and monocytes and in the other less characteristic lysosomal structures of additional blood and bone marrow cells [3 6 36 55]. Alkaline phosphatase [25] seems to be localized in the secondary or specific neutrophil granules which morphologically differ from the primary or azurophil granules containing acid phosphatase [3 6 7 56]. In neutrophils peroxidase [43] is probably localized in the same azurophil granules already mentioned as containing acid phosphatase [3 6 10 11 56]. In monocytes peroxidase was found to be confined to granules

specific for this cell type [8, 14] Comparative light microscopical studies yielded strong evidence that the Sudanblack-B positive granules in the neutrophils are really the same primary or azurophil granules referred to as containing both acid phosphatase and peroxidase [35, 54] and probably naphthol-AS-D chloroacetate esterase activity [37] This assumption is further supported by the cytochemical pattern of the Auer bodies, which are regarded as pathological forms of primary neutrophil granules, Auer bodies, in addition to acid phosphatase, also contain peroxidase and naphthol-AS-D chloroacetate esterase as well as Sudanblack-B-positive lipids [2, 15, 17, 27, 59]

Monocytes are characterized by prominent nonspecific esterase activity [30, 44], however, they also show a faint, but usually distinct, positive staining for peroxidase, chloracyl esterase, and Sudanblack B [47] The combined latter 3 cytochemical features as we have already stated are highly specific traits of neutrophils, and, excepting monocytes, are not shared by other blood or bone marrow cells The similarity of the cytochemical equipment possessed by these 2 cell types as well as the fact that the myelogenous origin of true blood monocytes [16, 29, 46, 48-57] has been unquestionably established seem to suggest a close genetical relationship between monocytes and neutrophils This suggestion is further supported by the observation of similar cytochemical and functional deficiencies occurring in both neutrophils and monocytes in some congenital affections of the neutrophil system (unpublished observations)

At the beginning of the sixties, cytochemical methods, based on the consistently characteristic patterns seen in normal blood and marrow cells, were employed as aids in identifying the various types of acute, or better named precursor cell leukemias [20, 27, 31] The peroxidase reaction had already been in use some decades as the established method for identifying immature myeloid leukemias

As just pointed out, most of these cytochemical procedures (table I) specifically stain subcellular structures and, therefore, may help in recognizing atypical cells which can otherwise hardly be identified In fact, the presence of such cytochemically stainable cellular structures, as for instance the primary granules in neutrophil precursors may be regarded as an unequivocal sign of a tendency toward specific cellular differentiation

Cytochemical methods also led to improved diagnostic detection of monocytic leukemia [9, 20, 29, 31, 47] Some of the cases, now identifiable with considerable certainty as belonging to the monocyte series, were in the past incorrectly diagnosed as cases of myeloblastic leukemia [47] The criteria that have proven to be most useful in diagnosing monocytic leukemia when ex-

Table 1 Cytochemical pattern of the leukemic cell populations in various types of leukemia

Reaction (ref.)	Myelo- blastic	Promyelo- cytic	Myelomonocytic	Monocytic	Erythremia	Lymphatic	Myeloma plasmacytic leukemia
Naphthol AS LC acetate esterase [44]	± - +	+ - + +	+ - + + +	+ - + + +	±	± - +	+ - + +
Naphthol AS LC acetate esterase + NaF (1.5 mg/ml) [44]	± - +	+ - + +	+ - + +	± - +	±	± - +	+ - (+ +)
α -naphthyl acetate esterase [44]	+ - + +	+	+ - + + +	+ - + + +	+ - + + +	+	+ - + + +
Naphthol AS Dechloro- acetate-esterase [37]	0 - (+)	+ - + + +	+ - + +	0 - +	0	0	0
Sudanblack B [54]	± - +	+ - + + +	+ - + + +	0 - + +	0	0	0
Peroxidase [43]	0 - +	+ - + + +	+ - + +	0 - +	0	0	0
PAS [21]	0 - ±	0 - +	+	0 - +	+ - + + +	± - + +	0 - +
Acid phosphatase [7]	± - +	+ - + +	+ - + +	+ - + +	+ - + + +	+	+ - + + +
Iron [13]	0	0	0	0	0 - +	0	0

† The techniques employed are based upon the methods published by Löfgren [30]

specific for this cell type [8, 14] Comparative light microscopical studies yielded strong evidence that the Sudanblack-B-positive granules in the neutrophils are really the same primary or azurophil granules referred to as containing both acid phosphatase and peroxidase [35, 54] and probably naphthol AS-D chloroacetate esterase activity [37] This assumption is further supported by the cytochemical pattern of the Auer bodies, which are regarded as pathological forms of primary neutrophil granules. Auer bodies, in addition to acid phosphatase, also contain peroxidase and naphthol-AS-D-chloroacetate esterase as well as Sudanblack-B-positive lipids [2, 15, 17, 27, 59]

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Reaction (ref.)	Myelo- blastic	Promyelo- cytic	Myelomon- ocytoid	Monocytic	Erythremia	Lymphatic	Myeloma plasma cell leukemia
Naphthol AS LC acetate esterase ³ [42]	± - +	+ - + +	+ - + +	+ - + +	±	± - +	+ - + +
Naphthol AS-LC acetate esterase + NaF (1.5 mg/ml) [44]	± - +	+ - + +	+ - + +	± - +	±	± - +	+ - (+ +)
n-naphthyl acetate esterase ⁴ [44]	+ - + +	+	+ - + +	+ - + +	+ - + +	+	+ - + +
Naphthol AS D-chloro- acetate-esterase [37]	0 - (+)	+ - + +	+ - + +	0 - +	0	0	0
Sudanblack B [54]	± - +	+ + - + +	+ - + +	0 - + +	0	±	0
Peroxidase [43]	0 - +	+ + - + +	+ - + +	0 - +	0	0	0
pAS [25]	0 - ±	0 - +	+	0 - +	+ - + +	± - +	0 - +
Acid phosphatase [7]	± - +	+ - + +	+ - + +	+ - + +	+ - + +	+	+ - + +
Iron [13]	0	0	0	0	0 - +	0	0

¹ The techniques employed are based upon the methods published by Löffler [30]

hibited as follows are: The presence of marked amounts of sodium fluoride (NaF) – and organophosphate – sensitive esterase splitting naphthol AS-acetates accompanied by relatively faint positive staining reactions with Sudanblack-B and for peroxidase as well as for chloracylesterase [1, 47]. In fact, all the cases identified by these means as monocytic leukemia behaved accordingly in the further fairly specific tests: (1) they showed an early and abnormally rapid monocyte emigration in skin window experiments [45] with consecutive transformation into macrophages [45], (2) when cultured *in vitro*, the leukemic cells transformed depending upon to the culture technique into large vacuolated macrophages or fibroblast-like cells [38, 39, 50], (3) in all cases showing predominantly mature cells the serum and urinary lysozyme levels were very high [4, 9, 40, 41], (4) in the few cases tested, the leukemic cells showed an IgG receptor activity [9] reported to be specific for the monocytes and macrophages [22].

The 4 criteria listed above plus the cytochemical pattern previously cited provide the most reliable methods for establishing the diagnosis of monocytic leukemia [9]. We had the occasion to study some cases which according to other authors [27, 29, 32] should be identified as monocytic leukemia but in our tests failed to show either the typical NaF-sensitive naphthol-AS-acetate-esterase as well as the characteristic lysozyme levels or the behaviour typically observed in the skin window experiments or in the *in vitro* cultivation. Based on the criteria reported above a further type of leukemia could be identified, which is characterized by a rather immature monocytic or promonocytic cell population [23, 52] which, however, clearly differs from the mature monocytic leukemia as well as from the myelo-monocytoid type or the myeloblastic and promyelocytic types.

Based upon cytochemical features, an atypical kind of leukemia could be identified which showed a pattern of differentiation falling in between that of the typical monocytic and the typical promyelocytic patterns [51]. Similar discrepancies were also observed in electron microscopic and in biochemical investigations [23, 51] giving further indications of the atypical maturation which we denoted 'myelomonocytoid' [51].

Although other bone marrow cells do not contain organelles which may be unequivocally cytochemically characterized, they do show when the appropriate cytochemical staining procedures are used, highly characteristic patterns, which may sometimes be distinctly altered in pathologic circumstances. For instance, in acute erythremias and erythroleukemias the leukemic erythroblasts exhibit very strong esterase splitting a naphthyl acetate for most part exceeding the enzyme activity observed in normal or otherwise

pathological erythroblasts. Further traits frequently encountered in leukemic erythroblasts include an increase in the normal acid phosphatase activity in a circumscribed paranuclear area, as well as increased iron content and marked PAS-positivity in the leukemic erythroblasts [1, 13, 28, 32].

Myeloma cells and leukemic plasma cells may be identified by the characteristically greater activities of both their paranuclear acid phosphatase and their α -naphthyl acetate esterase [1, 33, 42].

An other group of acute leukemias lacks any typical cytochemically demonstrable enzyme activity but is characterized by small or coarse granules of PAS positive materials [21]. The majority of these cells are uniformly small displaying a narrow cytoplasmic rim, in other cases an additional number of larger sized cells may be present. According to various authors [20, 27, 31] this particular kind of leukemia has been referred to as an 'acute lymphatic' or 'lymphoblastic' leukemia. However, the lymphatic origin of this type of leukemia has not been satisfactorily confirmed, and the term is based only on the similarity between the PAS-positive granules in this type of leukemia and those encountered in the lymphatic cells of chronic lymphatic leukemia and lymphosarcoma. Furthermore, the granular deposits of glycogen [35] are obviously not related to the subcellular structures linked to the identifying functional characteristics of these cells. Granular deposits of similar PAS-positive substances could be seldom observed in typical myeloblastic cells but are a well known occurrence in monocytic leukemia [35, 45]. Probably these deposits are in some way related to the metabolic features peculiar to these cells and, as it has been repeatedly noted by various authors, the amount of stainable glycogen may vary considerably during the course of the disease and may be particularly influenced by chemotherapy [12, 18, 24]. Nevertheless, the presence of PAS-positive granules in leukemic cells without any other characteristic cytochemical features has led to the distinction of a group of leukemias which are particularly inclined to respond favorably to glucocorticoid therapy and usually permit during their fatal course, repeated therapeutically induced remissions.

In respect to this behaviour these leukemias differ somewhat from those of the cytochemically completely 'undifferentiated' group. Although the members of the latter group may respond well to therapy, they commonly show a less favorable prognosis in childhood than the PAS-positive types [26]. The term 'cytochemically undifferentiated' refers to the lack of any characteristic cytochemical features in the leukemic cell population although very weak nonspecific esterase and acid phosphatase activities have been observed, as well as that of some oxidative enzymes [32].

In comparative studies the identification of acute leukemia based on cytochemical methods was found to be superior to the conventional morphological classification approach, in that the detection of the frequently distinct cytochemical traits allowed a reasonable classification of a given leukemic cell population, whose morphologic identification was otherwise either arbitrary or uncertain.

Furthermore, atypical features revealed by cytochemical methods may be encountered in the blood and bone marrow cells of patients with leukemia. A diagnostic value may be attributed to these cytochemically atypical cells similar to that of the well known abnormalities of cytoplasmic maturation and of nuclear morphology. For instance, atypical neutrophils showing a markedly reduced or a complete lack of Sudanblack-B staining, or of the peroxidase and the naphthol-AS-D chloroacetate esterase activities may be frequently observed in myeloblastic, myelocytic, myelomonocytic, monocytic and erythroleukemias [20]. An atypical increase in the enzyme activities in the cells may also be regarded as a sign of leukemic deviations from the normal physiological maturation. For instance, alkaline phosphatase, which is not present under normal conditions, was found in leukemic myeloblasts [34, 53], and considerably more naphthylamidase activity than usual was observed in abnormal leukemic neutrophils [49], leukemic monocytes repeatedly showed even greater nonspecific esterase activity than normal ones (unpublished observation).

Conclusions

Cytochemical staining procedures have been well established as additional aids in identifying the various types of acute leukemias. Some of the techniques employed are based on the demonstration of the presence in subcellular organelles of certain enzymes and chemical constituents which specifically characterize both the tendency and the degree of differentiation of the leukemic cell population. Using cytochemical criteria it is possible to specifically identify the various acute granulocytic leukemias, monocytic leukemia, myelodysplasia, and erythroleukemia as well as plasmacytoma and plasma cell leukemia. The granular PAS-positivity as a characteristic cytochemical feature of acute 'lymphatic' leukemia is discussed. A group of morphologically immature leukemias lacking sufficiently characteristic features is termed an 'undifferentiated type'. The cytological pertinency of this type is not yet clear.

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Studies on the Nature of Acquired Immunity Against Leukemia in Guinea Pigs¹

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Abstract Subcutaneous inoculation of leukemic cell suspensions of 10^{-2} to 10^{-6} concentration into 84 'strain 2' and F_1 hybrid guinea pigs produced subcutaneous leukemic infiltrations at the site of inoculation in 80 of the inoculated animals leading to a generalized stem-cell leukemia. When 96 'strain 2' and F_1 hybrid guinea pigs were inoculated intradermally, 67 of them developed intradermal tumors, 34 of these tumors (51%) regressed spontaneously. Twenty one females and 11 males in which the intradermal tumors regressed were reinoculated subcutaneously. Nineteen out of 21 females (90%) and 7 out of 11 males (64%) resisted the challenging inoculation suggesting thereby that the induced immunity was more pronounced in females than in males. The acquired immunity could not be transmitted to other guinea pigs by a serum collected from the immunized animals.

Key Words

Immunity against leukemia
Leukemia in guinea pigs
Leukemia virus

We have reported briefly in a previous study [9] that 'strain 2' or F_1 hybrid guinea pigs can be actively immunized against a transplantable strain of stem cell leukemia. The immunization procedure consists of intradermal inoculation of a very small dose of a diluted suspension of leukemic cells. A small intradermal leukemic tumor develops at the site of inoculation, in preliminary experiments previously reported, the intradermal tumors regressed spontaneously in 13 out of 31 guinea pigs (42%) leaving the animals immune to a challenging reinoculation of heavy doses of leukemic cell suspensions by any route.

The present study describes additional experiments dealing with the induction of active immunity in guinea pigs by intradermal inoculation of leukemic cell suspensions.

¹ Aided in part by grants from the Damon Runyon Memorial Fund and the American Cancer Society.

Materials and Methods

The L₂C leukemic strain which originated as a spontaneous leukemia in one of strain 2 guinea pigs [3] and which has been carried by cell graft in animals of this inbred line [9-11] was employed in this study. We have already mentioned in our previous study [9-10] that this strain of stem-cell leukemia is uniformly leukemogenic on cell graft transfer for strain 2 or F₁ hybrid guinea pigs and that it induces in these animals a rapidly progressing and uniformly fatal stem-cell leukemia.

Practically all strain 2 guinea pigs employed in this study were bred by brother-to-sister mating in our laboratory; a few were obtained from Horton's Laboratory Animals Inc. in Los Gatos, Calif. F₁ hybrid guinea pigs born in our laboratory to Hartley females and strain 2 males were also used.

Preparation of leukemic cell suspensions. A guinea pig with advanced leukemia was sacrificed by ether inhalation. After the skin was shaved the abdominal cavity was exposed. A fragment of the subcutaneous leukemic tumor from the site of inoculation, also small fragments of spleen, of the mesenteric tumor, and of an enlarged peripheral lymph node were removed aseptically, weighed, cut with scissors, and ground in a mortar. Sterile physiological saline solution being added to obtain a cell suspension of 10^6 concentration, the cell suspension was then passed through a voile cloth and poured into a sterile tube immersed in a small glass jar filled with ice cubes. Serial dilutions of desired concentration were then prepared from the original cell suspension and used immediately for inoculation.

Results

Subcutaneous Inoculations of Leukemic Cell Suspensions

Leukemic cell suspensions of 10^2 to 10^8 concentration were inoculated under the skin of the right flank into young adult 3 to 6-week old strain 2 or F₁ hybrid guinea pigs. Table I summarizes the results of these experiments. Fifty three out of 55 strain 2 guinea pigs inoculated subcutaneously with leukemic cell suspensions of 10^2 to 10^8 concentration developed leukemic cell infiltrations which appeared at the site of inoculation, grew progressively and led to a generalized and fatal stem-cell leukemia. Among the 16 strain 2 guinea pigs inoculated with 10^7 and 10^8 dilutions, only 3 developed leukemia; the remaining 13 animals did not react to the initial inoculation during an observation period extending from 5 to 8 weeks. In a parallel series, 32 F₁ hybrid guinea pigs were inoculated subcutaneously with leukemic cell suspensions of 10^6 to 10^{-7} concentration and 30 of them developed generalized leukemia.

Of the 15 strain 2 and 2 F₁ hybrid guinea pigs which did not react to the initial subcutaneous inoculation, 13 were reinoculated under the skin of the opposite i.e. left flank with leukemic cell suspensions of 10^2 con-

Table 1 Results of subcutaneous inoculation of leukemic cell suspensions into young adult 'strain 2' and F₁ hybrid guinea pigs

Strain	Leukemic cell concentration ¹	Number of guinea pigs		Leukemia incidence, %	Average latency, days	
		inoculated	developed leukemia		initial	terminal
'Strain 2'	10 ⁻²	24	24	100	14	22
	10 ⁻³	10	10	100	16	27
	10 ⁻⁴	8	8	100	24	45
	10 ⁻⁵	6	5	83	24	30
	10 ⁻⁶	7	6	86	27	36
Total 10 ⁻² to 10 ⁻⁶		55	53	96	-	-
'Strain 2'	10 ⁻⁷	14	3	21	34	39
	10 ⁻⁸	2	0	-	-	-
F ₁ hybrids	10 ⁻²	12	12	100	18	31
	10 ⁻³	6	5	83	14	20
	10 ⁻⁴	6	5	83	20	33
	10 ⁻⁵	1	1	100	28	36
	10 ⁻⁶	4	4	100	25	41
	10 ⁻⁷	3	3	100	28	37
Total 10 ⁻² to 10 ⁻⁷		32	30	94	-	-

¹ 0.3 to 0.5 ml each, inoculated under the skin of the right flank

centration, and 11 of them developed leukemic cell infiltrations at the site of reinoculation, which progressed rapidly into a generalized and fatal leukemia. The remaining 4 animals were challenged by intradermal reinoculation of a leukemic cell suspension of 10⁻³ concentration, as a result, 2 of them developed leukemia (table II).

Intradermal Inoculations of Small Doses of Leukemic Cells

In another series of experiments, small doses (0.1 ml) of leukemic cell suspensions, varying in concentration from 10⁻² to 10⁻⁷, were inoculated intradermally into young, adult, 3- to 6-week-old 'strain 2' or F₁ hybrid

Table II Susceptibility of guinea pigs that did not react to the initial subcutaneous inoculation to a challenging reinoculation of leukemic cells

Strain	Route of challenging inoculation ¹	Number of guinea pigs		
		inoculated	developed leukemia	remained negative
'Strain 2'	s.c.	12	10	2 ²
	i.d.	3	2	1
F ₁ hybrids	s.c.	1	1	0
	i.d.	1	0	1

¹ Subcutaneous challenging inoculation consisting of 0.3 to 0.5 ml of a 10^{-4} leukemic cell suspension. The intradermal challenge consisted of 0.1 to 0.2 ml of a 10^{-3} concentration of a leukemic cell suspension.

² Both guinea pigs received a second challenge consisting of subcutaneous inoculation of a leukemic cell suspension of 10^{-4} concentration and both developed leukemia.

guinea pigs. Among 82 inoculated animals, 56 developed intradermal tumors at the site of inoculation after a latency varying from 10 to 22 days.

Microscopic examination revealed that these small intradermal tumors did not actually consist of solid tumor masses, but were caused by a diffuse infiltration of the upper, and in some instances also of the deeper layers of the dermis, by clusters and strands of leukemic cells (fig. 1). The leukemic cells contained large numbers of virus particles (fig. 2).

Among the 56 intradermal leukemic tumors thus induced, 30 (54%) regressed spontaneously within 4-8 days after their appearance (table III). In the remaining animals, the intradermal tumors persisted for 1-2 weeks, then gradually increased in size, changed into solid tumor masses, metastasized to the adjoining lymph nodes, and eventually led to the development of a generalized leukemia.

In a similar, parallel series, 14 F₁ hybrid guinea pigs were inoculated intradermally with leukemic cell suspensions of 10^{-3} to 10^{-7} concentration and 11 of them developed intradermal tumors. The tumors regressed spontaneously in 3 out of 4 guinea pigs which received the 10^{-4} dilution, and in 1 animal inoculated with the 10^{-7} concentration. Thus, a total of 4 animals out of 11 recovered from the intradermal tumors (36%).

Under the conditions of our experiments, there was no apparent sex difference in the incidence of spontaneous regression of the intradermal tumors induced in the inoculated animals.

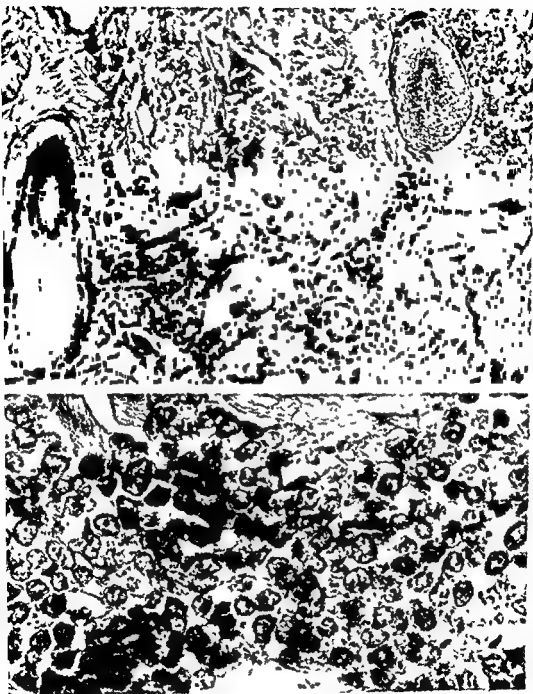
*Fig 1a-b*

Table III Results of intradermal inoculations of small doses of leukemic cell suspensions into "strain 2" guinea pigs

Leukemic cell concentration	Number of guinea pigs		Average time i d tumors developed, days	Number i d tumors regressed	Incidence i d tumors regressed, %	Average time i d tumors regressed, days	Number of guinea pigs	
	inoculated	developed i d tumors					developed generalized leukemia	remained negative
10^{-3}	17	12	11	3	25	5	9	5
10^{-4}	29	25	13	17	68	8	8	4
10^{-5}	15	9	15	4	44	4	5	6
10^{-6}	13	10	20	6	60	6	4	3
10^{-7}	8	0	—	—	—	—	—	8
Total all dilutions	82	56		30	54			
Dilutions 10^{-4} to 10^{-7}	65	44		27	61			

In our previous studies carried out with a transplantable sarcoma on mice [5] a significant sex difference was observed in the natural resistance of the inoculated animals to the induced intradermal tumors. The small intradermal tumors regressed more frequently in females than in males. However, this difference was observed only in those animals in which intradermal tumors had been induced with high dilutions of tumor cell suspensions. Castration decreased the higher resistance of females and increased the resistance of males [6].

Some of the animals in which the intradermal tumors regressed were kept for further observation, a few were sacrificed for microscopic and electron microscopic studies. The remaining guinea pigs were submitted subsequently to a challenging reinoculation of leukemic cells.

Fig. 1 Photomicrograph of a fragment of an intradermal tumor which was induced in a young adult F_1 hybrid male by intradermal inoculation of 0.1 ml of a leukemic cell suspension of 10^{-4} concentration. The intradermal tumor appeared 14 days after inoculation, was removed the next day, sectioned, stained and examined. a Leukemic cells in the dermis infiltrating diffusely between collagen fibers and around hair follicles. H.E. $\times 150$. b Higher magnification showing mononuclear leukemic cells with large vesicular nuclei. There is a mitotic figure in the center. H.E. $\times 600$.

*Fig. 2*

Table IV Resistance of immunized¹ guinea pigs to subcutaneous reinoculation of leukemic cell suspensions, influence of sex on induced immunity

Sex	Strain	Number of immunized guinea pigs challenged s.c. inoculation ²	Number of guinea pigs developed leukemia	Leukemia incidence, %	Average latency leukemia developed days
Females	'strain 2'	19	2		
	F ₂ hybrids	2	0		
	Total	21	2	10	26
Males	'strain 2'	9	3		
	F ₂ hybrids	2	1		
	Total	11	4	36	33

¹ Those guinea pigs in which the intradermal tumors regressed were considered to be 'immunized'. Some of the immunized guinea pigs received a second intradermal inoculation (0.2 ml) of a leukemic cell suspension of 10^{-3} concentration (booster dose), prior to the challenging subcutaneous inoculation (see text, page 226)

² 0.5 ml of a leukemic cell suspension of 10^{-2} concentration inoculated subcutaneously

Resistance of animals in which the intradermal tumors regressed to challenging reinoculation of leukemic cells. Influence of sex on induced immunity. As already reported in our previous study [9], most of the guinea pigs in which the intradermal tumors regressed spontaneously, were resistant to a challenging reinoculation of heavy doses of leukemic cell suspensions by any route tested

Fig. 2 Electron micrograph of a fragment of a leukemic cell from an intradermal tumor. This tumor was induced in a F₂ hybrid, young, adult, male guinea pig by intradermal inoculation of 0.1 ml of a leukemic cell suspension of 10^{-4} concentration. The intradermal tumor appeared 14 days after inoculation and was removed the next day, embedded, sectioned and examined in the electron microscope $\times 68,000$. Many characteristic immature virus particles in the cisternae of the endoplasmic reticulum. The doughnut like particles are about 90 m μ in diameter, and have a thick, granular, rather fuzzy, outer coat. They are similar to those described in our previous study [4, 10]. Electron micrograph prepared by DOROTHY G. FELDMAN in our laboratory

In our current experiments here reported, the challenging reinoculations consisted of subcutaneous injections of 0.5 ml each of leukemic cell suspensions of 10^8 concentration (table IV). In a few instances, cell suspensions of 10 percent concentration were also inoculated.

There was a remarkable difference between the immunized males and females in their resistance to a challenging reinoculation of leukemic cells. Nineteen out of 21 immunized females (90%) resisted completely challenging reinoculations of heavy doses of leukemic cells, as compared with 7 out of 11 immunized males (64%). Four out of 11 males in which the initial intradermal tumors regressed, did not resist a challenging reinoculation of leukemic cells.

A similar observation on sex difference in resistance of immunized animals to a challenging reinoculation of tumor cells, was made previously in our earlier studies carried out on a transplantable mouse sarcoma [5].

Attempts to increase the resistance of the immunized animals by a second intradermal inoculation of leukemic cells (booster dose) Some of the guinea pigs in which the intradermal tumors regressed, received a second intradermal inoculation (0.2 ml) of a leukemic cell suspension of 10^8 concentration, prior to the challenging subcutaneous inoculation. Although additional experiments are needed to evaluate the enhancing effect of the booster dose on the resistance of the immunized animals, results of preliminary experiments thus far performed, suggest that a second intradermal inoculation increased the degree of acquired immunity.

When 10 immunized females that had received a booster dose were challenged by a subcutaneous inoculation of leukemic cells, none developed leukemia, whereas out of 9 immunized guinea pigs that had received a booster dose, 2 developed leukemia following a similar challenge. In a parallel experiment carried out on males, 2 out of 7 immunized animals that had not received a booster dose, developed leukemia following a subcutaneous challenge, as compared with 1 out of 2 immunized males challenged without a prior booster inoculation.

Susceptibility of guinea pigs which did not react to the initial intradermal inoculation, to a challenging reinoculation of leukemic cells Among the guinea pigs inoculated intradermally with small doses of leukemic cell suspensions, some did not react to the initial inoculation. These animals were then reinoculated with leukemic cell suspensions. It is evident from table V that among such animals, some were resistant to reinoculation of heavy doses of leukemic cells. The results appeared to be influenced by the route of the challenging inoculation and the dose employed. Thus, among 13 guinea pigs

Table V Susceptibility of guinea pigs which did not react to the initial intradermal inoculation, ■ a challenging reinoculation of leukemic cells

Strain	Route of challenging inoculation ¹	Number of guinea pigs		
		inoculated	developed leukemia	remained negative
Strain 2	intradermal	12	3	9
F ₁ hybrids	intradermal	1	0	1
Subtotal		13	3	10 ¹
*Strain 2	subcutaneous	■ 13	13	
F ₁ hybrids	subcutaneous	2	2	
Subtotal		20	15	5 ²

¹ Subcutaneous challenging inoculation consisted of 0.3 to 0.5 ml of a 10^{-2} leukemic cell suspension. The intradermal challenge consisted of 0.1 to 0.2 ml of a 10^{-2} concentration of a leukemic cell suspension.

² Of the 15 guinea pigs which remained negative after the first challenging inoculation, 13 were again reinoculated (5 intradermally and 8 subcutaneously) and 1 guinea pig in each of these groups developed leukemia.

challenged by intradermal inoculation of 0.1 to 0.2 ml of a 10^{-2} dilution of leukemic cells, only 3 developed leukemia. Among those challenged by subcutaneous inoculation of 0.5 ml of a 10^{-2} dilution of leukemic cells, 15 out of 20 developed leukemia.

The question remains to be answered why those guinea pigs which did not visibly react to the initial intradermal inoculation of leukemic cells, developed an immunity. It is possible to assume that such animals developed small intracutaneous leukemic cell infiltrations, which remained unnoticed, and subsequently regressed without trace.

We have stressed in our previous communication [9] that recognition of small intradermal tumors, which may develop following intradermal inoculation of very small doses of leukemic cells, may be difficult. In some instances, such tumors may be barely visible as a small, slightly elevated, round thickening of the skin about 2-3 mm in diameter, may persist for only a few days and then disappear without trace. Such small intradermal infiltrations may be readily missed, unless the animals are examined carefully on a daily basis and their hair is closely clipped.

It is possible, therefore, that we could have missed a few small intradermal tumors in some of the animals observed, and that, consequently, among the guinea pigs that had been inoculated intradermally and were thought not to have reacted to the initial inoculation, there were some that actually did develop very small tumors which subsequently regressed, leaving such animals immune to a challenging reinoculation.

In our previous studies carried out on mice we have repeatedly made the observation that animals which did not react to the initial intradermal inoculation of tumor cells and which did not develop, and recover from, intradermal tumors, were not rendered immune [7, 8].

Attempts to Transmit Resistance from Immunized Guinea Pigs by Immune Serum

An attempt was made to determine whether the immunity which developed in those guinea pigs in which the intradermal tumors regressed, could be transferred passively to other guinea pigs by a serum collected from such animals.

Two 'strain 2' guinea pigs in which the intradermal tumors regressed spontaneously and which did not react to a challenging reinoculation of leukemic cell suspensions were bled. Sera obtained from blood collected from these animals were inactivated at 56 C for 30 min, distributed and stored in ampoules at -20°C, and then used within a few weeks. Normal sera were at the same time obtained from blood collected from 2 healthy 'strain 2' guinea pigs, inactivated at 56 C for 30 min, then stored at -20 C and used in a control series. Immune serum from the first animal was employed in experiments 1 and 2, the other immune serum was used in experiment 3.

In the first experiment leukemic cell suspensions of 10^{-2} concentrations were mixed each with an equal volume of 10^{-1} , 10^{-2} , and 10^{-3} dilutions of immune serum. In a simultaneous control experiment the leukemic cell suspensions were mixed with similar dilutions of a normal guinea pig serum. The mixtures were incubated for 60 min at room temperature, followed by incubation at refrigerator (+4 C) temperature for 75 min, they were then inoculated subcutaneously (1 ml each) into young adult 'strain 2' guinea pigs. Three guinea pigs were inoculated with the leukemic cell immune serum mixtures and 2 with leukemic cell normal serum mixtures, all 5 animals developed leukemia after 12-14 days.

In a second experiment, leukemic cell suspensions of 10^{-3} and 10^{-4} concentration were mixed each with an equal volume of a $1/10$ dilution of immune serum. In a control experiment, the leukemic cell suspensions of equal concentration were mixed with a similar dilution of a normal guinea pig serum. The mixtures were incubated for 1 h at room temperature followed by an additional incubation for 3 h at refrigerator (+4 C) temperature. Two F₁ hybrid guinea pigs were inoculated subcutaneously (1 ml each) with the leukemic cell immune serum mixtures and 2 control animals were inoculated with leukemic cell normal serum mixtures. All 4 guinea pigs developed leukemia after 2-3 weeks.

In the third experiment, leukemic cell suspensions of 10^{-4} and 10^{-5} concentration were mixed, each with an equal volume of a 1:1 dilution of immune serum. In a control experiment, leukemic cell suspensions of equal concentration were mixed with a similar dilution of a normal guinea pig serum. The mixtures were incubated for 1 h at room temperature, followed by 3 h at refrigerator ($+4^{\circ}\text{C}$) temperature. Two F_1 hybrid guinea pigs were inoculated with the leukemic cell immune serum mixtures and 2 control animals were inoculated with the leukemic cell normal serum mixtures. All 4 guinea pigs developed leukemia after 3 weeks.

The results of these preliminary experiments suggested clearly that neither of the serum had any apparent neutralizing effect on the leukemogenic potency of the leukemic cells. Under the conditions of our experiments, immunity observed in those guinea pigs in which the intradermal tumors regressed could not be transferred by serum collected from such animals, to other guinea pigs. A similar observation was previously made in our studies carried out with BESREDA on a transplantable epithelioma in rabbits [1] and on the Rous sarcoma in chickens [2].

Acquired immunity not transmitted to offspring The animals which developed, and recovered from, intradermal tumors, did not transmit the acquired immunity to their offspring. This was illustrated in the following experiment.

Strain 2 female No 40 and 'strain 2' male No 56 were inoculated intradermally with leukemic cell suspensions of 10^{-4} and 10^{-5} dilution respectively. They both developed intradermal tumors, which regressed spontaneously. Both animals received intradermal booster inoculations and later 2 successive challenging subcutaneous inoculations of heavy doses of leukemic cells. They remained in good health and were now considered to be immunized.

Female No 40 was then mated to male No 56. Their first litter consisted of 1 female and 2 males; the second litter, born 2 months later, consisted of 3 females and 2 males. When these F_1 offspring reached the age of about 6 weeks, they were challenged. 2 were inoculated intradermally with 10^{-2} dilutions and the remaining 6 were inoculated subcutaneously with 10^{-2} dilutions of leukemic cell suspensions. They all developed local leukemic tumors at the site of inoculation, which enlarged progressively and led into a generalized and fatal stem-cell leukemia.

Conclusions

Subcutaneous inoculation of leukemic cell suspensions of 10^{-2} to 10^{-4} concentration into either 'strain 2' or F_1 hybrid guinea pigs induced in practically all inoculated animals local leukemic infiltrations, which progressed gradually and led to a generalized and fatal stem-cell leukemia.

None of the subcutaneous leukemic tumors induced at the site of inoculation regressed spontaneously

When higher dilutions of leukemic cell suspensions were injected some of the animals did not react to the initial inoculation. Such animals did not develop immunity, when challenged by a second inoculation of a sufficient dose of leukemic cells. Most of them developed, and died from, generalized leukemia.

Intradermal inoculations of very small doses of leukemic cell suspensions resulted in the development, at the site of inoculation, of small intradermal leukemic infiltrations, which regressed spontaneously and eventually disappeared in over 50% of the inoculated animals.

Those animals in which the intradermal tumors regressed, were in most instances resistant to a challenging subcutaneous reinoculation of leukemic cells. This resistance was more pronounced in females than in males. Ninety per cent of the immunized females resisted reinoculation of heavy doses of leukemic cells as compared with only 64% of males. The resistance of the immunized animals could be increased by intradermal reinoculation of a small booster dose of leukemic cells.

In several experiments it was noticed that a majority of those guinea pigs which did not apparently react to the initial *intradermal* inoculation of small doses of leukemic cells, proved to be resistant to a challenging subcutaneous reinoculation of heavy doses of leukemic cell suspensions. It is possible that at least some of these animals developed very small intradermal tumors which remained unnoticed, and then regressed without trace leaving such animals immune to a challenging reinoculation of leukemic cells.

Attempts to transmit resistance of the immunized guinea pigs to other animals by an immune serum have not been successful. Under conditions of experiments thus far employed immunity observed in those guinea pigs in which the intradermal tumors regressed, could not be transferred by a serum, collected from such animals, to other guinea pigs.

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Serum Iron-Binding Capacity in Pregnancy

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Abstract Although iron binding capacity increases during pregnancy, there is no correlation of it with mean values of serum iron and haemoglobin levels. The administration of drugs (vitamins or vitamins + iron) do not influence either mean haemoglobin, serum iron levels or iron binding capacity.

Key Words

Iron binding capacity
Pregnancy
Serum iron

It has long been known that pregnancy causes anaemia the nature of which is not so clear. A low haemoglobin level may be due to hydraemia observed in pregnancy, levels, however, lower than 12 g/100 ml are compatible with true anaemia [1]. The anaemia of pregnancy was attributed at first to iron balance disturbances. In recent years detailed studies have demonstrated that other factors also, such as vitamin B₁₂ and folic acid deficiencies contribute to its appearance. Although the responsible factors are known, the relative role played by them in the causation of anaemia of pregnancy is not easy to estimate because more than one factor appear to operate. Also the incidence of different types of anaemia in pregnancy vary greatly geographically [7].

This investigation constitutes part of a study of anaemia among Greek pregnant women, and was carried out in order to determine the incidence of iron deficiency among these subjects. The subjects were not selected and we do not know whether any of them had an iron deficiency before pregnancy. Also 40% of them received vitamins.

Material and Methods

Unsaturated and total serum iron binding capacity were measured in 250 pregnant women at various stages of pregnancy.

Table 1 Total iron binding capacity (TIBC) unsaturated iron binding capacity (UIBC) haematocrit (Hct) haemoglobin (Hb) and serum iron in pregnant and non pregnant women

Weeks of pregnancy	No of cases	TIBC $\mu\text{g}\%$		UIBC $\mu\text{g}\%$		Hct %		Hb g%		Serum iron $\mu\text{g}/100\text{ ml}$	
		\bar{x}	$\pm\text{ISD}$	\bar{x}	$\pm\text{ISD}$	\bar{x}	$\pm\text{ISD}$	\bar{x}	$\pm\text{ISD}$	\bar{x}	$\pm\text{ISD}$
10-20	31	425	87	324	63	38.9	3.2	12.2	1.53	91	32.1
21-30	111	443	79	379	96	37	3.6	11.8	1.42	79	31.7
31-41	156	471	93	408	82	38.3	4.13	11.7	2.48	79	37.2
Non pregnant	20	342	118	246	162.7	41.06	2.64	13.9	1.3	96.6	23.8

Haemoglobin and haematocrit determination and erythrocyte counts were also carried out. Unsaturated iron binding capacity (UIBC) was estimated by an isotopic method using Irosorb-59 (Abbott radio-pharmaceuticals). The procedure goes through the following steps: (1) mixing of the patient's serum with the radioactive iron, (2) addition of the Irosorb, (3) radioassay of the above mixture plus Irosorb, (4) Irosorb repeated rinsing with distilled water, (5) radioassay of the sponge. The results are expressed as unsaturated iron binding capacity.

$$\frac{(\text{Net original cpm}) - [(1.05) \times (\text{Net sponge cpm})]}{\text{Net original cpm}} \times \mu\text{g Fe added} \times 100 = \mu\text{g Fe}/100\text{ ml}$$

In 220 of the cases, total iron binding capacity (TIBC) was estimated by determining the serum iron.

Results

According to the weeks of gestation, the results are shown in table 1. The mean value of the haemoglobin does not show any significant change during pregnancy, while the mean serum iron level falls but not below normal values (table 1).

The pregnant women were also divided into 3 groups according to their haemoglobin level: group A, with haemoglobin level lower than 10 g% had a TIBC of 450 $\mu\text{g}\%$, group B, with haemoglobin level between 10 and 12 g% had a TIBC of 454 $\mu\text{g}\%$, and group C, with haemoglobin level higher than 12 g% had a TIBC of 462 $\mu\text{g}\%$.

Discussion

There is an increase in unsaturated and total iron binding capacity throughout pregnancy, more pronounced in UIBC. This is in agreement

with the results of other investigators [3 7 9] Statistical analysis of these results showed that there is a significant difference only in the mean values of UIBC between the pregnant women in the 10th to 20th week and in the 31st to 41st week of gestation ($p = 0.001$)

In pregnancy there is a need for iron thus early mobilization of storage iron occurs [4] This mobilization is achieved by an increase in iron binding capacity which is probably caused like in other serum binding capacities by an increase production of oestrogen [2, 5] Besides oestrogen low serum iron level is also considered to act as a stimulus for iron binding capacity increase [4] In the present study it is supposed that the first mechanism rather exists since UIBC and TIBC increase though serum iron levels remain within the normal ranges

No correlation was found between the haemoglobin level and total iron binding capacity The steady level in haemoglobin concentration observed in this study can be explained by the increase of red cell volume which follows the increased plasma volume [6 8]

It should be noticed that 40% of the studied women received vitamins and sometimes a combination of vitamins plus iron But despite this there is no differences of iron binding capacity serum iron and haemoglobin level between women who received the above drugs and those who did not

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Microspectrophotometric and Electron Microscopic Studies of Bone Marrow in Hereditary Sideroblastic Anaemia

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Abstract Ultrastructural studies in 3 patients with hereditary sideroblastic anaemia have shown iron laden mitochondria in a proportion of the non dividing, late polychromatic erythroblasts. In contrast to the situation in primary acquired sideroblastic anaemia, the mitochondria of the proliferating erythroblasts were rarely infiltrated with iron, and a combined quantitative cytochemical and autoradiographic study showed no significant arrest of cell proliferation. It is likely that impaired haemoglobin synthesis is more important than ineffective erythropoiesis in the pathogenesis of the anaemia in this disease.

Key Words

Autoradiography
Cytochemistry
Erythropoiesis
Electron microscopy
Mitochondria
Sideroblastic anaemia

Electron microscopic studies of erythroblasts in primary acquired sideroblastic anaemia [2, 11, 14, 16], and the secondary sideroblastic anaemias [5, 6] have shown abnormal accumulations of iron within the mitochondria. Hereditary sideroblastic anaemia is rare, and ultrastructural studies have been described in only a single case by HEILMEYER *et al* [8-10]. In this patient, mitochondrial iron deposits similar to those seen in the non-hereditary sideroblastic anaemias were present.

This paper describes the ultrastructural features of the erythroblasts in 3 further cases of hereditary sideroblastic anaemia. In addition, haemopoietic cells have been studied by the technique of combined quantitative cytochemistry and autoradiography to determine whether there is any evidence of intramedullary cell death during the proliferative phase of erythropoiesis. The patients studied were 3 members (cases 1, 2 and 8) of the family described by LOSOWSKY and HALL [12], in which 10 out of 110

members in 4 generations were affected. The disease showed a well marked, sex-linked partially recessive inheritance.

Methods

Marrow aspirations were made from the sternum and a portion of the aspirate was incubated in heparinised Hanks' solution containing 10 μ Ci tritiated thymidine (3 H TdR)/ml (specific activity = 5 000 Ci/M) at 37°C for 30 min prior to the preparation of smears. The distribution of the various haemopoietic cells in the different stages of interphase (G_1 , S and G_2) was determined as described previously [17-19].

The remainder of the marrow aspirate was immediately fixed in 4% glutaraldehyde in 0.1 M Sorenson's buffer for 1 h at 4°C, for electron microscopy. The marrow fragments were washed in the same buffer, post fixed for 1 h in buffered 1% osmium tetroxide, dehydrated and embedded in Araldite. Sections were cut on a Huxley microtome (Cambridge Scientific Instruments Ltd) using glass knives, and mounted on uncoated grids. The sections were double-stained with uranyl acetate and lead citrate and examined in an AEI 6 M electron microscope.

Results

Under the light microscope, all 3 samples of bone marrow showed a normoblastic erythroid hyperplasia, with an increased proportion of basophilic cells. Ringed sideroblasts were frequent, and this abnormality was seen predominantly in the non-dividing, late polychromatic erythroblasts. Numerous siderocytes were also present. Myelopoietic cells and megakaryocytes appeared normal.

The electron microscopic findings were qualitatively similar in all 3 patients. Ferritin molecules were observed in the cytoplasm of erythroblasts as dispersed granules, aggregates of varying size, and within micropinocytotic vesicles and larger vacuoles. A similar distribution of ferritin is seen in normal erythroblasts, but in the sideroblastic marrows some erythroblasts showed an increase in the number of dispersed ferritin molecules. Mitochondria

contained material resembling the iron micelles described by Bressis and Breton-Gorius [1]. The cristae of the affected mitochondria were less distinct than normal and the abnormal material was selectively deposited between the cristae. Sometimes the mitochondrial infiltrates consisted of

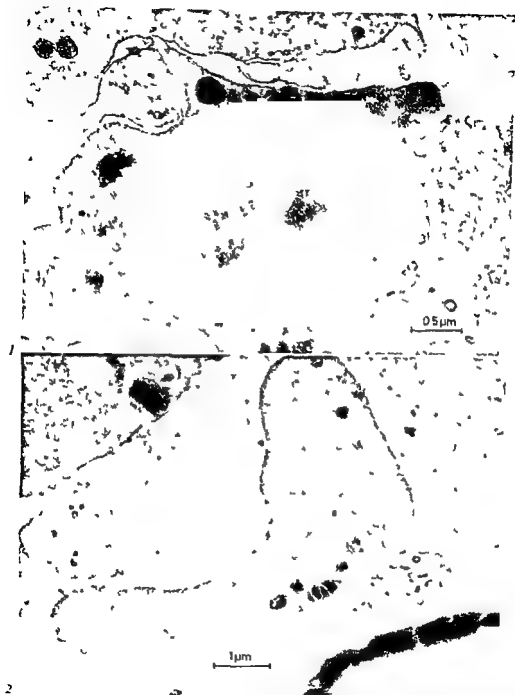


Fig. 1 Electron micrograph of late erythroblast showing iron laden mitochondria (case 1)

Fig. 2 Marrow siderocyte from case 8 showing iron laden mitochondria and aggregates of membrane bound iron which are probably mitochondrial remnants

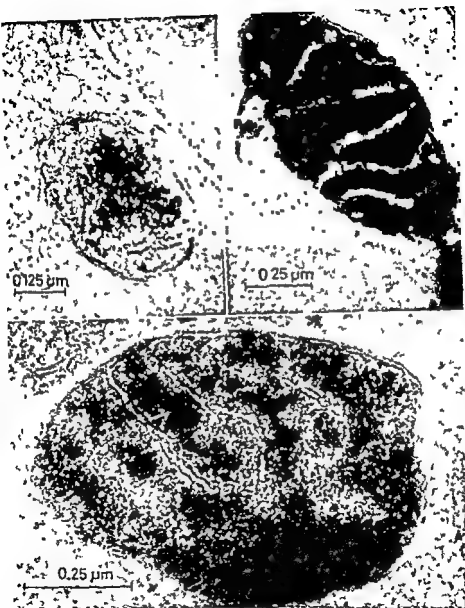


Fig. 3 Details of varying appearances of iron-containing mitochondria in the erythroblasts of hereditary sideroblastic anemia (case 2) *a* The granules are very coarse and resemble the dispersed ferritin molecules seen in the adjacent cytoplasm *b* and *c* The infiltrates often finely granular

Table 1 Distribution of haemopoietic cells in the various stages of interphase in hereditary sideroblastic anaemia

Cell type	Case	Percentages				S/G ₂	Number of nuclei assessed
		G ₁	S	G ₂	U		
Basophilic erythropoietic cells	1	14	81	5	0.4	17.5	260
	2	21	73	II	0	12.5	325
	8	18	73	8	0.7	9.8	268
Normal values		24-36	58-70	3-7	0-0.9	9.2-25.5	831
Early polychromatic cells	1	16	77	II	1	12.7	297
	2	20	71	II	1	8.8	299
	8	21	74	3	1.4	22.4	212
Normal values		4-14	77-85	4-10	0-0.7	8.3-21.2	868
Promyelocytes and myelocytes	1	65	29	II	II		138
	2	74	23	2	0.5		215
	8	65	33	2	0.7		149
Normal values		61-70	25-34	3-5	0-1.2		683

G₁ = Post mitotic cells with a diploid (2n) DNA content

S = Cells synthesising DNA

G₂ = Post synthetic, pre mitotic cells with tetraploid (4n) DNA contents

U = Cells with DNA contents lying between the 2n and 4n values which were not labelled with ³H TdR

compact accumulations of coarser granules which could have been ferritin. Not all of the late erythroblasts showed these abnormalities, a proportion had normal-looking mitochondria and some mitochondria only had a few scattered ferritin molecules within them.

Table I shows the distribution of the various proliferating haemopoietic cells in the different stages of interphase. Apart from an increased percentage of basophilic erythropoietic cells in S, and a concomitant decrease in the percentage in G₁, the results do not deviate appreciably from normal. In view of the difficulty in distinguishing early polychromatic cells in G₁ from non-dividing, late polychromatic cells (which also have a diploid DNA content), the slight increase of early polychromatic cells in G₁ is probably not significant [3].

Discussion

The ultrastructural studies reported in this paper establish that the siderotic granules seen under the light microscope in hereditary sideroblastic anaemia are qualitatively similar to those seen in the primary acquired type. These iron-containing granules correspond to mitochondria filled with a finely granular, electron-dense substance. Accumulations of iron within the mitochondria were not present in the early erythroblasts unlike the findings in primary acquired sideroblastic anaemia [6-11]. Although small deposits of mitochondrial iron were seen infrequently in the intermediate stages, iron laden mitochondria were usually seen in the late non-dividing stages only. The significance and chemical form of this iron-containing material have been discussed previously [2, 5].

Quantitative cytochemical and autoradiographic studies in 4 cases of primary acquired sideroblastic anaemia have shown a disturbance in the progress of early polychromatic cells through interphase [3, 18]. The two abnormalities detected were an increase in the number of early polychromatic erythroblasts in G_2 relative to the number of these cells in DNA synthesis, and an increase in the number of cells which were apparently arrested after a period in DNA synthesis ('U' cells). Precise kinetic conclusions cannot be drawn from such an analysis of a single marrow aspirate (table II). However, as the proportion of cells in G_2 was highest in those early polychromatic erythroblasts with the largest accumulations of stainable iron and because only a small proportion of early polychromatic ring sideroblasts were found to be in DNA synthesis, the abnormalities were

Table II Possible explanations for the abnormalities detected in primary acquired sideroblastic anaemia

Reduced S G_2 ratio	U cell
1 Arrest in G_1 leading to cell death	1 Arrest after period in DNA synthesis (permanent, temporary)
2 Prolongation of G_2 mitosis follows	2 Gross slowing of DNA synthesis
3 Prolongation of III with proportionately greater prolongation of G_2	3 Aneuploidy
4 Shortening of S	4 Loss of DNA from arrested G_2 cell
	5 Decrease in thymidine kinase activity

attributed to an arrest of proliferation caused by the pathological accumulation of iron within these cells. In support of this hypothesis was the electron microscopic demonstration of iron laden mitochondria in the early and late erythroblasts in this disease [6, 11], and the fact that mitochondrial damage is known to produce a G₂ arrest [4].

In the 3 cases of hereditary sideroblastic anaemia there was no 'pile up' of basophilic or early polychromatic erythroblasts in G₂ and only a small number of 'U' cells were present. Therefore, unlike the primary acquired form, in hereditary sideroblastic anaemia, cell death in the proliferative phase of erythropoiesis must be either absent or minimal. This may be due to differences in the distribution of erythroblast iron in the hereditary and the primary acquired types [7]. In the hereditary type, ringed sideroblasts occur predominantly in the non-dividing, late erythroblasts, whereas in the primary acquired type ringed sideroblasts occur at all stages of erythroblast maturation (proliferating and non-proliferating).

The high ³H-TdR labelling index in the basophilic erythropoietic cells in hereditary sideroblastic anaemia may be due to (1) a shortening of interphase with either a normal S period or a proportionately smaller reduction of S, (2) a prolongation of S with a normal duration of interphase, or (3) a prolongation of S with a proportionately smaller prolongation of interphase. In β thalassaemia major, where a similar finding has been reported proliferation in the basophilic cells appears to be increased with a considerable shortening of both interphase and the S period [20].

This investigation excludes the presence of a major arrest of erythropoietic cell proliferation in hereditary sideroblastic anaemia, but it is still possible that a proportion of non-dividing late polychromatic cells with extensive mitochondrial damage may die within the marrow. Radioactive iron studies in this disease have shown a normal or increased plasma iron turnover, and the red cell iron utilisation has usually been moderately or markedly reduced, suggesting the possibility of intramedullary cell death [9, 12, 15]. However, ferrokinetic studies cannot distinguish between impaired haemoglobin synthesis with re-cycling of excess non-haem iron, and ineffective erythropoiesis [18]. The blood picture in hereditary sideroblastic anaemia shows a more striking hypochromasia and a lower MCHC than in the primary acquired form [13]. It is therefore likely, that the contribution of ineffective haemoglobin production and ineffective cell production to the pathogenesis of the anaemia in these 2 conditions is different, with ineffective haemoglobin production being the major component in the hereditary form and ineffective cell production in the primary acquired type.

Acknowledgements We wish to thank the 3 members of the family studied for their cooperation and Mrs JANE PRATT for valuable technical assistance. This work was supported by a grant from the Yorkshire Council of the British Empire Cancer Campaign for Research.

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Normal Human Red Cells Treated *in vitro* with Cephalothin

A Scanning Electron Microscope Study

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Abstract Normal human erythrocytes treated *in vitro* with cephalothin were studied by scanning electron microscope. On the surface of the majority of the erythrocytes numerous tiny projections, uniform in size and length were observed. Some cells in the center or in the rim presented a few round and large protuberances. It is suggested that the morphologic abnormalities can be ascribed to an alteration of the cell membrane, already demonstrated with serological and enzymatic investigations.

Key Words

Cephalothin treated erythrocytes
Electron microscopy
Erythrocyte deformation
Surface ultramicroscopy

It has recently been shown that normal human red cells treated *in vitro* with the antibiotic cephalothin under suitable experimental conditions develop a positive direct Coombs test [11] and became abnormally sensitive to the lytic action of complement [17]. Moreover acetylcholinesterase, which is located in the outer surface of the erythrocyte, is markedly reduced [6, 7], similarly the oxygen uptake in the presence of methylene blue, which reflects the membrane metabolism, is subnormal [6]. All these findings indicate that a lesion of the erythrocyte membrane occurs as a consequence of the *in vitro* treatment with the drug.

In order to evaluate whether the above serologic and enzymatic abnormalities have a morphological counterpart, treated cells were studied with the transmission and scanning electron microscope. The aim of the present paper is to report the results obtained.

Materials and Methods

Blood drawn with acid-citrate dextrose from 4 normal adult individuals was centrifuged at 1 500 rpm for 10 min and the plasma and the buffy coat were removed by suction. The

erythrocytes were washed with saline 3 times and an aliquot treated with cephalothin (Keflin[®], Lilly) as previously described [17]. Another aliquot was treated in the same way except the exposure to cephalothin and used as a control. After the last washing with saline both control and treated erythrocytes were fixed in 2% phosphate buffer glutaraldehyde (pH 7.4) for 30 min at 4°C. After a triple rinse in distilled water for 30 min the erythrocytes were resuspended in distilled water. One drop of this suspension was placed and dried on a supported disk. After coating with gold platinum in a Hitachi vacuum chamber, the specimens were examined in a Cambridge "Stereoscan" electron microscope at an angle of 45° at 20 kV, using Tri X Kodak film.

An aliquot of treated cells were fixed with cold 2% glutaraldehyde buffered at pH 7.4 with Millonig's solution [10], briefly rinsed, postfixed with phosphate buffered 2% OsO₄, rinsed with Ringer's solution and treated with 0.5% cold uranyl acetate [5]. Dehydration in ethanol and propylene oxide and embedding in Epon 812 were carried out following routine procedures. Thin sections stained with uranyl acetate and lead citrate were examined under a Hitachi H 11 A electron microscope at 75 kV.

Results

The examination of treated cells with the transmission electron microscope did not show any significant abnormality, in particular, the erythrocyte membrane maintained its classical triple-layered appearance. Vacuoles, inclusion bodies, ferritin granules were not observed. On the contrary the aspect of treated red cells under the scanning electron microscope was markedly different from that of the control specimens. The majority (90%) of the erythrocytes had lost their normal biconcave shape, assuming an irregular globular appearance (fig. 1). The surface was characterized by numerous tiny projections, uniform in size and length (fig. 1-3). Some cells did not show such striking changes, however they too appeared abnormal since either in the center or in the rim they presented a few round and large protuberances (fig. 1, 4).

Discussion

Serologic [11, 17] and enzymatic [6, 7] investigations have produced evidence that cephalothin can cause an alteration of the red cell membrane. The present study confirms this assumption through a different approach. Two kinds of red cell alterations have been found under the scanning electron microscope: a minor percentage of cells showed a few round protuberances on the surface and most of them the striking finding of numerous tiny projections.

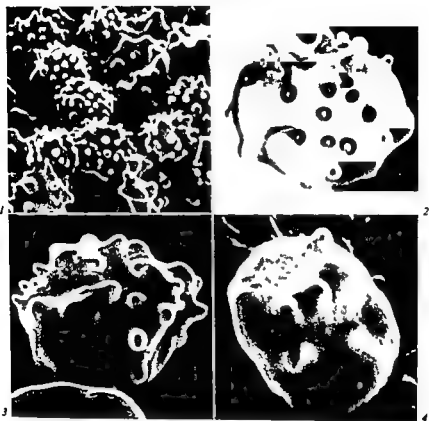


Fig 1 Cephalothin treated erythrocytes at low magnification $\times 1600$

Fig 2 and 3 Cephalothin treated erythrocytes with an irregular globular appearance and numerous tiny projections $\times 8700$

Fig 4 Cephalothin treated erythrocyte with a few round and large protuberances $\times 8700$

The interpretation of the above findings is not easy. The two kinds of lesion may be the expression of a cell damage of different degree, perhaps reflecting a different sensitivity of the erythrocytes to the drug. Although not so numerous as in cephalothin treated erythrocytes, protuberances have been described in various conditions: newborn [12] and splenectomized subjects [12], patients with autoimmune haemolytic anaemia [14] and paroxysmal nocturnal haemoglobinuria [9]. Moreover protrusions have been observed in

erythrocytes treated *in vitro* with sodium fluoride to inhibit glycolytic capability and produce deficiency of ATP, that is essential for the maintenance of normal membrane structure and deformability [1]

On the other hand, a morphologic picture characterized by so numerous tiny projections has been observed in other conditions. BESSIS *et al* [2, 3, 8] reported that washing with saline can determine projections in normal red blood cells, they however disappear after glutaraldehyde fixation. Following BESSIS *et al* [3], their persistence after fixation is an expression of abnormality of the cell membrane. In fact projections have been described in α -beta lipoproteinemia [8]. The above data suggest that the morphologic abnormalities observed in cephalothin-treated erythrocytes can be ascribed to an alteration of the cell membrane. This conclusion invites comparison with other cells carrying a membrane lesion that we had the opportunity to investigate with the scanning electron microscope, i.e. paroxysmal nocturnal haemoglobinuria (PNH) erythrocytes and normal red cells treated with the sulfhydryl compound AET (2-aminoethylisothiuronium bromide) (PNH-like cells). Both types of cells, similarly to cephalothin-treated erythrocytes, are abnormally sensitive to the lytic action of complement [13, 15, 16, 19] and display low acetylcholinesterase activity [4, 18]. However the aspect of PNH and AET red cells is different from that of cephalothin-treated erythrocytes, in fact PNH red cells present protuberances, pits and craters on the surface [9] and AET cells appear completely deformed and show pronounced abnormal depressions (unpublished observations). Although differences in appearance could reflect artifacts verified during specimen preparation rather than a different membrane alteration, the above suggests that cells that give similar serologic reactions can have a completely different morphologic appearance under the scanning electron microscope.

Acknowledgments We thank Prof. E. BALDACCI, Director of Istituto di Patologia Vegetale dell'Università di Milano, for access to the Cambridge Stereoscan electron microscope, and Mr. B. QUARONI for the technical assistance.

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Haemoglobin K Woolwich ($\alpha_1\beta_1$ 132 Lysine \rightarrow Glutamine) in Ghana

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Abstract A fast moving haemoglobin, K Woolwich was found in patients admitted to Korle Bu Teaching Hospital, Accra, and in members of 2 tribes which live in different regions of Ghana and differ linguistically and ethnically. Although haemoglobin K Woolwich is the third most common abnormal haemoglobin in Ghana, it has not yet been found except in these tribes. This is possibly due to the difficulty in detecting the haemoglobin variant in the presence of haemoglobin A. It would be interesting to examine the amino acid sequence of cases of 'haemoglobin K' reported from other parts of West and North Africa in order to determine whether they are cases of haemoglobin K Woolwich. This may throw light on genetic links and migration routes of tribes in West Africa.

Key Words

Abnormal haemoglobins
Haemoglobins in Ghana
Haemoglobin K Woolwich

Haemoglobin K is a 'fast' haemoglobin which moves between haemoglobins A and J on electrophoresis at pH 8.6. It was first discovered in Algerian Berbers [4] and since then a number of other examples have been found in Africa, for instance in Upper Volta [20], Liberia [19] and Portuguese Guinea [23]. Examination of the haemoglobin structure showed that there were a number of haemoglobins K with different amino-acid substitutions but similar electrophoretic mobilities. For example, K Ibadan (β 46 Gly \rightarrow Glu) and K Woolwich (β 132 Lys \rightarrow Gln) are β -chain variants [2] whilst the haemoglobin K described in an East Indian family [1] probably has a substitution in the α -chain.

In a survey carried out in Northern Ghana by EDINGTON and LAING [7] among the Dagomba, 3 blood samples were found with a haemoglobin

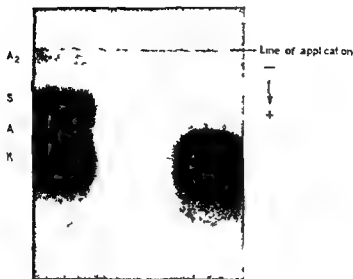


Fig 1 Paper electrophoresis pH 8.9 Hb A and K on the right do not separate

fraction moving faster than haemoglobin A identified by AGER and LEHMANN [1] as haemoglobin K. In Ghana we have now found 24 people from 11 families with haemoglobin K. In one case each from 4 different families the haemoglobin has been identified as K Woolwich in which the 132nd amino acid residue of the 146 residues of the β -chain is one of glutamine instead of lysine as in haemoglobin A.

Material

Paper electrophoresis is carried out routinely on samples taken from patients admitted

Electrophoresis and Family Studies

On paper electrophoresis at pH 8.9 the fast moving haemoglobin was seen just ahead of haemoglobin A but there was no clear separation. When

Haemoglobin K Woolwich ($\alpha_1\beta_2$ 132 Lysine \rightarrow Glutamine) in Ghana

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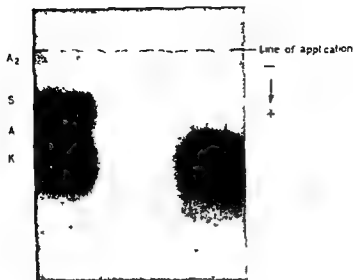


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Material

Paper electrophoresis is carried out routinely on samples taken from patients admitted to certain Wards of Korle Bu Teaching Hospital Accra the total number of samples being between 5 and 6 thousand annually. Also in March 1970 we went to Mpraeso and Abetifi two towns belonging to the Kwahu tribe (see fig. 7) and as part of a larger survey collected 163 samples from secondary schools and from a Nurses' Training College.

Electrophoresis and Family Studies

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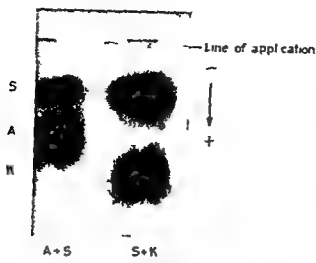


Fig 2 Paper electrophoresis pH 8.9 Separation of Hb A from Hb S

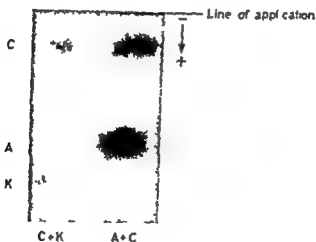


Fig 3 Paper electrophoresis pH 8.9 Separation of Hb A from Hb C

this haemoglobin was found not with haemoglobin A but with haemoglobins B or C its detection was easier. For example when A + S was compared with K + S it was possible to see clearly that in the latter case the non S fraction had a greater mobility than haemoglobin A (fig 1, 2 and 3). Table I gives some data about the samples in which the fast haemoglobin was found and figures 4 and 5 are family trees showing segregation of A, S and K.

Table 1 Hb K Woolwich in Ghana

Hb K Woolwich found	Number	Total number of samples
a) In hospital samples between November 1969 and April 1970	9	5 000-6 000 per year
b) In samples taken from students belonging to the Kwahu tribe	2	163
Tribal origin of the propositi with Hb K Woolwich		
a) Kwahu	7	
b) Busanga	4	
Haemoglobin type found in propositi		
a) AK	7	
b) SK	4	
Total number of relatives investigated	24	
a) AK	10	
b) CK	1	

There were no morphological changes in the red cells of individuals with A+K S+K heterozygotes did not show classical sickle-cell anaemia and the child with C+K did not have a modified haemoglobin C disease. In 2 of the A+K heterozygotes osmotic fragility tests were carried out and the results were within the normal range. In the S+K heterozygotes the ratio of S:K was about 3:2 but there was too little material in the C+K sample to make accurate measurements though there was clearly more C than K.

Identification of the Fast Haemoglobin

Electrophoresis in starch gel containing 6 M urea [5] showed that there was an abnormal β -chain present moving in a position which indicated one additional negative charge or one fewer positive charge. The haemoglobin was isolated by chromatography on DEAE Sephadex with a pH gradient [10] followed by paper electrophoresis at pH 8.9 [6]. The fingerprints (fig. 6) were prepared and stained [21] and showed only traces of the tyrosine containing peptide $\beta^A\text{TpXIII}$ ($\beta 121-132$) and the histidine containing peptide $\beta^A\text{TpXIV}$ ($\beta 133-144$). A new peptide was present which stained for both tyrosine and histidine. This suggested that the haemoglobin was haemoglobin K Woolwich $\beta 132$ (H10) Lys \rightarrow Gln [2] in which there is no tryptic hydrolysis at

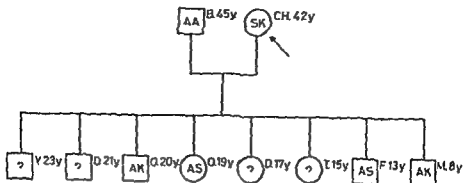


Fig. 4

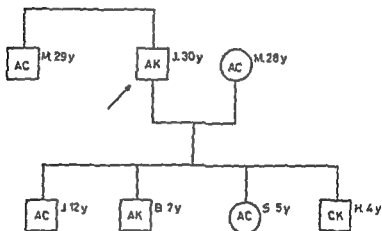


Fig. 4 and 5 Family trees, segregation of Hb A, K, S and C

Table II Amino acid sequence β 121-144 of the β -chain of human haemoglobin

	β TpXIII					β XIV		
Residue number	121	125	130	132		135	140	144
β -Chain of Hb A	Glu	Pro	Tyr	Lys	†	Ala	Ala	Lys †
β -Chain of Hb K	Glu	Pro	Tyr	Gln		Ala	Ala	Lys †

The arrows show points of tryptic hydrolysis. In Hb A 2 tryptic peptides are formed because the bond after lysine 132 is broken by trypsin.

In Hb K, residues 121-144 form a single tryptic peptide because the lysine in position 132 is substituted by glutamine.

Hb. K Woolwich

from Ghana

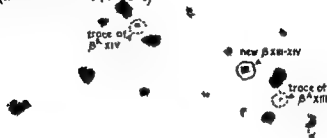
(traces of Hb A₂ present)

Fig 6 Fingerprint (peptide chromatogram) of the tryptic peptides of haemoglobin K Woolwich. Electrophoresis was carried out at pH 6.4. Tryptic peptides β^A XIII and XIV are present only in traces. These are due to the contamination of the Hb K by Hb A₂ which has the same electrophoretic mobility. The new peptide β^K XIII-XIV is indicated. For details see text and table 1.

position β 132 as there is no lysine, and peptides XIII and XIV are replaced by β^K XIII-XIV (table II).

The abnormal peptide was eluted from preparative fingerprints, hydrolysed and then analysed [3]. In all 4 cases amino acid analysis of the abnormal peptide showed that the composition was that of residues β 121-144 but with only one lysine and with an additional glutamic acid. The electrophoretic mobility of the haemoglobin and of the abnormal peptide (fig. 6) indicated the loss of only one positive charge and, therefore, the lysine must have been substituted by glutamine and not by glutamic acid.

Discussion

Two abnormal haemoglobins are common in Ghana, haemoglobins S and C [7, 8, 17, 22]. Some rare haemoglobins have also been found, but only in single families, G Accra [13], D Punjab [18], Korle Bu [11] and Osu-Christiansborg [12]. Haemoglobin K Woolwich was thought to be a rare variant, having been found only in one West Indian family [16] and in the Ivory Coast [CABANNES, CHARLESWORTH, PRICE and LEHMANN, unpublished].



Fig 7 Map of Ghana showing the places where the 2 tribes, the Kwahu and the Busanga, live

The combination of haemoglobins S and K Woolwich described by O'GORMAN *et al* [16], caused a mild anaemia and the ratio of S : K was similar to that in our cases. However, we did not find any symptoms of disease in people with the genotype S + K. It now seems that haemoglobin K Woolwich is more common than was thought, and is the third most frequent abnormal haemoglobin in Ghana. In the present study, all the cases of haemoglobin K Woolwich were found in members of 2 tribes, the Kwahu and the Busanga, which are widely separated geographically (fig 7). The language of the 2 tribes is quite different, the Kwahu belonging to the Akan and the Busanga to the Mande language group. According to their tradition, accepted by some historians [15, 24], the Akan people came from the river bend between Djenné and Timbuktu and were forced to migrate southwards at the end of the 13th century. They settled first in Northern Ghana where the Gonja tribe now lives, and then moved further south to their present territory, the Kwahu plateau. This is the only stretch of inhabited highland in Ghana, which is

protected on the West by the Kwahu Scarp, on the North-East by the Afram river, on the East by the Volta, and on the South by Akwapim Hills. Geographical isolation favours inbreeding, with an increase in the incidence of rare genes. The Busanga are part of the Mande group who formed the kingdom of Mali, which was at the peak of its power in the 14th century. Other members of the group can now be found in Mid-Sahara, Niger and Upper Volta.

The occurrence of haemoglobin K Woolwich in both tribes could suggest that they come from a common stock, and it is noteworthy that for both connections can be traced to present day Mali. However, it is difficult to reconcile this with their language difference and with the apparent lack of haemoglobin K Woolwich in tribes known to be more closely related to either of these two.

The exact incidence of haemoglobin K Woolwich both in general and in these 2 tribes cannot be assessed from our studies. According to a Population Census [9] in 1960 there were about 132,000 Kwahu and 56,000 Busanga in Ghana. From finding a number of unrelated cases in hospital patients and of 2 in Kwahu students it seems that haemoglobin K Woolwich is quite prevalent. The reason that it has not been found more frequently in Ghana, and not at all in the United States [14] is probably that haemoglobin K Woolwich is easily overlooked in the presence of haemoglobin A.

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The Effect of Certain Lipid Substances on Sickling

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Abstract Fifty individuals with sickle cell disease were given either steroids (testosterone for males and progesterone for females) at a dose of 10 mg or arachis oil intramuscularly. Sickling with metabisulphite was assessed at daily intervals for 5 days after the administration of the drug. It is concluded that these steroids inhibit sickling for a few days and that arachis oil inhibits sickling transiently and less profoundly whilst physiological saline did not affect sickling. The significance of daily variation in the generation of crises is discussed.

Key Words

Arachis oil
Progesterone
Sickle cell disease
Steroid hormones
Testosterone

The manifestations of sickle cell disease are ultimately explicable on the basis that the diminished solubility of haemoglobin S in environments with lowered oxygen tension results in sickling, and the distorted shapes produced either block the flow of blood to various organs or are sequestered off for destruction by the reticulo-endothelial system. Various workers have administered drugs of various types to Hb SS individuals in an endeavour to reverse sickling [1, 2, 3]. ISAACS and HAYHOE [4] observed a reversion of sickled erythrocytes to a normal contour *in vitro* in the presence of either testosterone, progesterone or durabolin. Samples of blood from 3 patients with sickle cell disease also proved to be resistant to sickling with sodium metabisulphite after administration of these steroids and it was hoped that these agents might be useful in the treatment of sickle cell disease. It was not certain at that stage whether the effect is limited to a few individuals or whether it is of general application. A double blind controlled study was planned to investigate whether there is any difference between the effect on sickling of an oily preparation of testosterone or progesterone and that of arachis oil (the base in which the steroids are dissolved).

Material and Methods

Fifty subjects (including children and adults of both sexes) with haemoglobin genotypes SS SC or S/thalassaemia were used in this study. Each male subject chose randomly either testosterone or oil and each female subject chose progesterone or oil.

1 ml of the solution chosen was injected intramuscularly (the steroids being at a concentration of 10 mg/ml) and samples of blood were taken immediately after and at 24 hourly intervals following the injection for 5 days. The metabisulphite sickling test [5] was performed on each sample. The sickling preparations (consisting of sealed slides of deoxygenated blood samples) were scored 1 h after they were made to determine the percentage of total erythrocytes sickled.

Results

The results are shown in tables I, II, III and summarised in table IV. The proportion of cells sickled on different days are compared using the

Table I Percentage of erythrocytes sickled after incubation for 1 h with 2% sodium metabisulphite. Testosterone injected group

	1st day	2nd day	3rd day	4th day	5th day	6th day
	83	25	8	-	7	-
	81	84	77	90	94	-
	73	82	-	14	23	23
	68	11	33	19	35	-
	68	77	63	81	74	90
	88	-	55	30	75	100
	53	21	25	53	-	-
	90	84	43	45	-	28
	70	88	30	79	31	80
	64	73	16	12	23	20
	60	96	30	74	42	24
	94	93	40	13	30	14
	94	90	51	51	34	-
	53	50	23	30	28	-
	33	27	30	16	38	16
	90	96	35	73	-	42
	72	81	81	53	24	36
	50	88	46	-	91	-
	40	77	16	46	27	63
	40	73	75	20	42	83
Σ	69.2	69.9	40.9	44.4	42.2	47.6
n	20	19	19	18	17	13

Table II Percentage of erythrocytes sickled after incubation for 1 h with 2% sodium metabisulphite. Oil injected groups (males and females)

	1st day	2nd day	3rd day	4th day	5th day	6th day
	62	46	8	13	96	87
	54	90	24	21	73	66
	40	84	53	86	67	72
	26	76	60	96	25	81
	66	60	2	16	31	64
	48	72	35	30	43	62
	40	32	72	-	28	58
	70	24	22	62	31	71
	66	2	38	16	36	69
	62	70	24	22	42	63
	56	60	36	25	4	30
	81	26	42	12	30	66
	50	20	14	26	44	-
	42	4	45	74	81	-
	38	94	61	30	42	-
	98	81	-	52	26	-
	82	46	96	91	61	-
X	57.8	50.1	37.6	40.7	44.7	60.7
n	17	17	16	16	17	12

Student's *t* test and the results are summarized in table V. It will be seen that the mean percentage of cells sickled in the oil injected groups is only significantly lowered on the 3rd and 4th days, when compared with the mean of the 1st day. However, in the testosterone and progesterone treated groups, there is a clear difference between the mean proportion of the cells sickled on the 1st day and the means of the 3rd, 4th, 5th, and 6th days respectively. Over the 5 days, the depth of depression of sickling was also more with the steroids than with oil. When a group of 12 sickle cell disease patients were tested using physiological saline (table VI) in place of the oil and steroids, there were no significant differences found between sickling tendencies on various days.

Discussion

It would appear, therefore, that whereas there is only a transient diminution in the proportion of cells sickled following the administration of

Table III Percentage of erythrocytes sickled after incubation for 1 h with 2% sodium metabisulphite Progesterone injected groups

	1st day	2nd day	3rd day	4th day	5th day	6th day
75	97	87	51	32	~	
86	90	7	32	39	~	
88	83	62	17	14	~	
83	77	18	23	22	~	
90	40	63	23	74	~	
78	80	30	20	23	~	
45	57	56	25	43	~	
42	28	30	10	14		60
90	76	41	53	70		40
56	52	17	14	23		36
75	34	2	20	28		40
46	34	42	51	32		23
2	34	73	86	88		26
\bar{X}	65.2	60.2	40.6	32.7	37.1	37.5
n	13	13	13	13	13	6

Table IV Proportion of red cells sickled at intervals after drug administration

	Testosterone	Oil	Progesterone
1st day	69.2 ± 8.5	57.6 ± 9.2	65.8 ± 14.0
2nd day	69.9 ± 12.6	52.1 ± 13.8	60.2 ± 13.0
3rd day	40.9 ± 9.6	37.6 ± 11.8	40.6 ± 14.0
4th day	44.4 ± 12.1	40.7 ± 13.8	32.7 ± 11.1
5th day	42.2 ± 12.0	44.7 ± 11.1	37.1 ± 11.1
6th day	47.6 ± 16.6	60.7 ± 9.2	37.5 ± 9.7
Maximum depression of sickling	Δ 41.6 ± 10.7	23.2 ± 15.2	38.2 ± 14.4

Population means for a 95-percent confidence limit Means $\pm 2 \times$ standard error

1 ml each of either 10 mg/ml of testosterone proportionate in oil, 10 mg/ml of progesterone in oil and 1 ml of oleum arachis II P was injected intramuscularly into each patient and samples of blood were obtained by fingerprick immediately after and at 24 hourly intervals after the injections. Vaseline-sealed slides of 2% metabisulphite treated cells were scored after one hour to derive the percentage of cells sickled.

¹ Δ is the percentage drop in sickling tendency over the duration of the study on each individual, where the change is maintained for at least 2 days.

Table V Comparison of sickling on day 1 with each subsequent day using Student's *t* test of significance

Student's <i>t</i> test to compare mean sickling for day 1 with each subsequent day ¹	Testosterone			Oil			Progesterone		
	<i>t</i>	De- gree of free dom	<i>p</i>	<i>t</i>	De- gree of free- dom	<i>p</i>	<i>t</i>	De- gree of free dom	<i>p</i>
<i>t</i> ₁₋₂	0.09	37	>0.15 <0.20	0.9	35	>0.15 <0.20	0.6	24	>0.475 <0.4875
<i>t</i> ₁₋₃	4.5	37	<0.0005	2.7	34	>0.005 <0.01	2.6	24	>0.005 <0.01
<i>t</i> ₁₋₄	3.4	36	<0.0025 >0.0005	2.1	34	>0.0125 <0.025	3.6	24	>0.0005 <0.0025
<i>t</i> ₁₋₅	3.7	35	<0.005	1.8	34	>0.025 <0.05	3.2	24	>0.0005 <0.0025
<i>t</i> ₁₋₆	2.3	31	>0.0125 <0.025	0.5	29	>0.30 <0.35	3.3	17	>0.0005 <0.0025

$$\text{Student's } t = \frac{X_1 - X_2}{\sqrt{SE_1^2 + SE_2^2}}$$

$$\begin{array}{llll} \text{1 } t-o & 1.99 & 34 & >0.25 \\ \delta & & & <0.05 \end{array} \qquad \begin{array}{llll} p-o & 1.44 & 28 & >0.05 \\ \delta & & & <0.1 \end{array}$$

*t*₁₋₂ etc. = Student's *t* test to compare the means for day 1 with day 2.

t-*o* and *p*-*o* } *t* test to compare the drop in sickling achieved with the hormones
 δ δ } testosterone and progesterone as compared with oil

As has been previously mentioned, this effect represents 'average' behaviour in erythrocytes of sickle cell disease patients in response to administration of these steroids. However, it should be emphasized that approximately 10% of subjects fail to respond to these steroids with any significant diminution in the proportion of cells which sickle in environments with lowered oxygen tension.

This study also shows that erythrocytes from each sickle cell individual vary from day to day in their ability to sickle. It has been suggested that

Table VI Percentage of erythrocytes sickled after incubation for 1 h with 2% sodium metabisulphate Physiological saline-treated group (n=12)

	1st day	2nd day	3rd day	4th day	5th day
68	68	70	■	70	
20	86	80	-	74	
82	64	82	87	86	
96	96	78	74	76	
78	76	74	84	80	
97	85	93	94	97	
83	87	80	-	-	
98	64	94	98	44	
96	48	98	19	-	
75	78	95	12	82	
96	85	85	45	94	
94	96	90	99	80	
Σ	81 92	77 75	85 33	69 50	78 30

selective destruction of erythrocytes in sickle cell disease with a high HbS content might add to this variability [6]. However, this variation might also be due to the effect of a substance (or substances) situated interiorly or exteriorly to the red cell membrane which influences the ability of the membrane to change in response to intracellular crystal formation in situations of lowered oxygen tension. Preliminary results from experiments in which cells from patients on high sickling days were mixed with plasma from the same patients on low sickling days and vice versa suggest that the ability of cells to sickle is influenced by plasma factors. The variation in levels of plasma factors and the variation in sickling ability which is associated would explain why sickling 'crises' sometimes occur and other times not in individuals with a constant amount of HbS.

A project separating sickling into 2 parameters viz (a) proportion of cells already sickled *in vitro*, (b) rate of sickling of the unsickled cells *in vitro* in response to deoxygenation is in progress to determine which of the 2 factors is more important in the development of crises. Blood gas studies have shown that differences occur between sickle cell disease patients and normal individuals with respect to pO_2 and pCO_2 in the blood [7]. Studies are in progress to determine whether the effect of the steroids on sickling is mediated through changes in these blood gas parameters, or if an effect

on arteriovenous pO_2 difference might cause a change in the metabolism of the erythrocyte membrane resulting in a change in its resistance to deformation by intracellular crystals. Plans are also in progress to determine what effect the administration of these steroids will have on sickling crises and how their continued administration will affect the incidence of recurrent crises.

Acknowledgements I am very much indebted to Prof H K. ADANOVON who supplied patients and technical help and criticized the scripts and to Dr E. LESI who also allowed me to study his patients. I am also grateful to Mrs M. OLUWOLE for supplying drugs, Miss O. OYEROLU and Mr J. AJAYI for technical assistance, Prof E. O. DOSEKUN for laboratory facilities and Prof L. LUZZATTO for criticizing the script. Dr J. W. K. DUNCAN carried out the statistical calculations and I am grateful to him.

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The Kinetics of Plaque-Forming Cells in Experimental Hypersplenism

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Abstract By i.p. application of methylcellulose (MC) hypersplenism has been induced in C57B1 mice. Decrease of red blood cells (RBC) and increase of reticulocytes and spleen index resemble the hemolytic anemia in man. No antibody formation against RBC of treated animals could be found. After sensitisation with sheep red blood cells a significantly impaired 19S- and 7S-antibody formation in single spleen cells could be demonstrated. These findings suggest an inhibition of macrophages by the uptake of MC.

Key Words

Antibody formation
Experimental hypersplenism
Macrophages
Methylcellulose
Mouse spleen
Plaque forming cells
Spleen cytology

By application of methylcellulose (MC) in rodents hypersplenism can be induced [2]. The histologic examination of the enlarged spleen shows a diffuse hyperplasia of the reticulum of the red pulp that was taken up by clusters of macrophages [6]. These cells enlarge also forming vacuols filled with MC [5, 9]. The half-life of red blood cells (RBC) is shortened and the bilirubin level is increased [5, 7, 8]. Removal of the spleen prevents from these phenomena. These findings resemble the hemolytic anemia in man. They let to suppose that the immune system and especially the spleen are involved by MC in any way. This was to demonstrate using the 'plaque technique'. Hereby single antibody-producing spleen cells can be detected after sensitisation with sheep red blood cells (SRBC). Spleen cells of sensitized animals are dispersed in gel among SRBC. Adding complement after incubation at 37°C for 90 min 19S-antibody producing 'direct plaque forming cells' (DPFC) are marked by local hemolysis in gel (LHG) around those cells. Adding anti-mouse globulin as 'developing serum' before complement 7S-antibody with low hemolytic activity producing 'indirect plaque forming cells' (IPFC) can be identified.

Plaque technique was also performed to demonstrate, if by LHG antibodies against RBC of treated animals can be detected

Material and Methods

Animals Female mice of the strain C57B1 have been used. Their weight varied about 30 g. Each animal was at least 4 months old.

Treatment with MC Powder was dissolved in warm distilled water and i.p. injected. Twice a week doses of 1 ml of a 5% solution were given until the total dose of 100 mg was reached.

RBC, white blood cells (WBC), reticulocytes and spleen index Ten animals have been sacrificed 2 days after the last single dose of MC. After differential staining RBC and WBC were counted. The spleen has been isolated from fat and connective tissue and weighed.

The quotient $\frac{\text{spleen weight}}{\text{body weight}} \times 100$ has been constituted (spleen index).

Controls were 10 untreated animals.

Demonstration of PFC The animals were separated into different groups. *Series A* was sensitized with 4×10^8 SRBC i.p. 1 week after the last dose of MC. At the 2nd, 3th, 5th, 9th, 10th, 14th and 21th day after the sensitisation (in the following day +2, +3 and so on) the spleen was removed. The 19S- and 7S-antibody producing cells were detected by LHG in the usual way [1, 3, 4]. Each spleen was examined separately. *Series B* were untreated mice but sensitized with 4×10^8 SRBC i.p. LHG was performed at the same points after sensitisation as series A. *Series C* consisted of MC-treated mice avoiding sensitisation. In this series LHG was performed using RBC of the treated animals.

Results

An early reaction of the animals to the application of MC could not be seen. Five weeks after the beginning of the treatment they became lethargic and weak. At the end of the treatment the enlarged spleen could be palpated through the abdominal walls. The sole pathological finding in the opened abdomen was the enlarged but in colour and consistency normal spleen. No ascites has been seen.

Changes of the hemogram After treatment with MC the RBC decreased from $8.96 \pm 0.49 \times 10^6$ to $7.58 \pm 0.64 \times 10^6/\text{mm}^3$ in untreated controls ($P < 0.001$). On the contrary the reticulocytes increased after treatment with MC from $3.8 \pm 1.0\%$ to $7.8 \pm 1.9\%$ in the controls ($P < 0.001$). The spleen index increased from 0.45 ± 0.09 to 1.06 ± 0.21 in treated mice ($P < 0.001$). The WBC showed no significant changes after treatment with MC.

Kinetics of PFC The kinetics of PFC were different in treated mice and controls. Without treatment with MC using the direct method the first DPFC

were demonstrated 48 h after sensitisation with SRBC. The peak value was reached at the day +5. After this point there was a rapid decrease. At the day +14 19S-antibody producing spleen cells rarely could be found. Using a developing serum the first IPFC were demonstrated at the day +5. At this point there were fewer 7S- than 19S-antibody producing spleen cells. After the day +9 this relation changed. Contrary to 19S-antibody producers 7S-antibody producers could be demonstrated until day +21.

After treatment with MC the kinetics of PFC principally were similar. The content of PFC was, however, reduced at each point. A significant diminution ($P < 0.001$) could be stated regarding the DPFC at the days +3, +5 and +9, whereas the IPFC were diminished significantly ($P < 0.001$) at the days +5, +9 and +10.

The effect of MC has been seen especially at the day +5, when the peak value was reached with regard to the 19S-antibody producing cells. The quotient between treated and untreated animals was at this point 1/10. This quotient never was reached at other days. The maximal quotient in refer to 7S-antibody producing cells was only 1/5. The quotient between treated and untreated animals approached to 1/10 with increasing distance from the sensitisation with SRBC.

After a single dose of MC and sensitisation with SRBC no difference to untreated could be found regarding the DPFC and IPFC. Using RBC and spleen cells of treated mice no PFC could be demonstrated. 19S-antibody producing cells per 10^6 spleen cells of treated mice and controls are compared in table I. 7S-antibody producing cells per 10^6 spleen cells of treated mice and controls are compared in table II. Time diagrams of DPFC and IPFC of treated mice and controls are shown in figures 1 and 2.

Table I 19S antibody producing cells per 10^6 spleen cells in MC-treated mice and untreated controls

Days after sensitisation	Untreated animals	Treated animals	Significance #
2	0, 0	0, 0	
3	7, 5	0, 0	<0.001
5	237, 211	29, 27, 27, 16	<0.001
9	9, 8	3, 3	<0.001
10	2, 2	3, 3	
14	2, 0	1, 4, 2, 0	
21	0, 0	0, 0, 0	

Table II 7S-antibody producing cells per 10⁶ spleen cells in MC-treated mice and untreated controls

Days after sensitisation	Untreated animals	Treated animals	Significance P
2	0, 0	0, 0	
3	1, 0	0, 0	
5	96, 67	18, 17, 16, 11	<0.001
9	64, 58	17, 10	<0.001
10	33, 33	8, 5	<0.001
14	19, 19	10, 3, 2, 1	
21	8, 6	3, 1, 0	

Discussion

In these experiments the changes of the hemogram as described by other investigators could be confirmed. The treated animals shows besides the decrease of RBC an increase of the reticulocytes. Spleen index increases in the same manner. Moreover, it can be demonstrated that the treatment with MC interferes with the kinetics of antibody producing cells in the spleen. In relation to the controls PFC are reduced in treated animals. This is true in all experiments at different points after sensitisation with SRBC for 19S-antibody producing cells as well as for 7S-antibody producing cells. A significant impairment of PFC after treatment could be demonstrated only at the peak of the cellular antibody formation. But this is sufficient to state an influence of MC on the immune response. However, the different counts of PFC at other points allow an interpretation in which way MC may have interfered with the immunologic system.

Using the direct method PFC can be found in treated mice 5 days after the first sensitisation whereas the controls have already DPFC after 3 days. Both groups reach the peak value at the day +5. Animals treated with MC have about 10% regarding the count of DPFC in controls. Later the reduction of DPFC is not so striking and at last there is no difference between series A and series B. Principally the same is true using the indirect method for detection of PFC. Treated mice have those cells at the day +5, untreated at the day +3 already. At the maximum value in treated mice the reduction of 7S antibody producing spleen cells is about 20%. Later on the reduction is not so apparent but contrary to the DPFC always below controls.

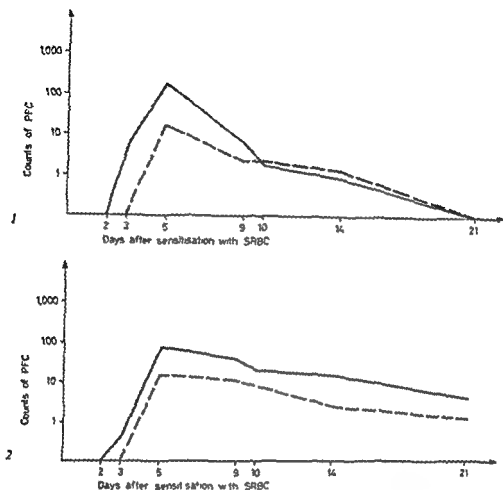


Fig 1 Time-diagram of 19S-antibody producing spleen cells in treated mice and controls (mean values) — Controls ——— Treated mice

Fig 2 Time-diagram of 7S-antibody producing spleen cells in treated mice and controls (mean values) — Controls ——— Treated mice

These findings point to an impairment of the immunologic response in respect to antibody forming spleen cells. This could be explained by aggregation of SRBC by MC. This would mean a reduction of antigenic determinants situated at the surface of the SRBC. But this explanation is not sufficient because it could be shown that the content of PFC is not impaired after one single dose of MC.

An other explanation for this impairment is the inhibition of the macrophages. The uptake of MC as it could be demonstrated histologically causes a blockade of the incorporating cells as it is known for other inert macro-

molecules. This means a diminished capacity of processing the SRBC. Thus less information is passed to the antibody forming cells. This interpretation gives an explanation for the maximum impairment at the peak value of the immune response.

Another possible mode of action of the MC is the inhibition of the plasma cells. This would also reduce the antibody formation. But this effect would reduce the PFC at each point after the sensitisation.

Using spleen cells and RBC of treated mice for the LHG neither 19S-antibody nor 7S-antibody forming cells could be found. This means that there is no hemolysis induced by an antigen-antibody reaction. A direct effect of the MC on the RBC inducing a specific antibody formation must be denied.

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Haemoglobin D-Thalassaemia

Remarks on the article of R. C. JAIN, H. S. ANDLEIGH and J. B. MEHTA, Acta haemat 44 124-127 (1970) If the father had homozygous haemoglobin D-disease, how could one explain that the propositus' brother did not have haemoglobin D with the exception of illegitimacy? However, if it is assumed that the father also has haemoglobin D- β -thalassaemia it should be shown by the family studies, or at least by whether haemoglobin A₂ determinations in the father, mother and the propositus were raised.

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Hacettepe University, Ankara (Turkey)

Reply to the above remarks of Prof. ÖZSOYLU The father had homozygous haemoglobin D, the mother had β -thalassaemia, the propositus showed haemoglobin D-thalassaemia, and the brother of the propositus showed only β -thalassaemia. It is possible, and can be explained by the child having received only genes from the mother, resulting only in β -thalassaemia. However, the levels of haemoglobin A₂ were not raised in this case nor in a few other cases of β -thalassaemia (unpublished observation). SUKUMARAN *et al* [1] reported a case of haemoglobin D-thalassaemia, where the father showed only haemoglobin D with 17% alkali-resistant haemoglobin. The mother had thalassaemia, one of the 4 children showed haemoglobin D-thalassaemia while 3 children revealed only thalassaemia. In another study WEATHERALL [2] observed a woman with haemoglobin C-thalassaemia, who had a daughter with the same condition. The father of the child showed no evidence of thalassaemia or any abnormal haemoglobin. However, extensive blood grouping could not exclude paternity. This case confirms the close linkage between the β -structure and the β -thalassaemia loci.

Dr. RATAN JAIN M. D., Department of Pathology and Bacteriology,
Dr. S. N. Medical College, Jodhpur (India)

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Österreichische Gesellschaft für Hämatologie

Vom 24. bis 26. 9. 1971 findet in Graz die 2. Österreichische Hämatologentagung statt. Das wissenschaftliche Thema lautet: Immunglobulinveränderungen und Blutkrankheiten.

Den Kongress betreffende Anfragen sind zu richten an Prof. Dr. H. KALOD, Univ. Kinderklinik, Landeskrankenhaus, A-8036 Graz (Österreich).

On the Origin of Human Basophilic Granulocytes

M R PARWARESCH, L-D LEDER and K E G DANNENBERG

Institute of Pathology, University of Kiel (Director Prof Dr E LENNERT)

Abstract By a combined application of toluidine blue stain and naphthol AS-D chloroacetate esterase reaction on normal bone marrow smears metachromatic and promyelocytic granules were demonstrated on the same cell. Precursors of basophils contain NASDCI-esterase activity, the intensity of which depended on the maturity of the cells. All transitional cells were found between non metachromatic promyelocytes with strong NASDCI-esterase activity and completely enzymatically negative metachromatic segmented basophils. It is concluded that basophilic granulocytes like monocytes, neutrophils, and eosinophils derive from non specific promyelocytes. Evidence has been presented for the transition of promyelocytic granules to specific basophilic granules. Our results confirm the dualistic view of the cytogenesis of leukocytes.

Key Words

Basophilic leukocytes
Bone marrow
Cytochemistry
Leukocyte cytogenesis
Mast cells
Metachromasia

PAUL EHRLICH [4] was the first to identify blood basophils in leukemias. A few years later it was shown that the basophilic granulocyte is a normal blood constituent. It was considered to be a special type of granulocyte which is formed in the bone marrow. This was supported by occurrence of mast myelocytes in the bone marrow [5], mitotic activity of mast myelocytes [5, 11], demonstration of peroxidase activity in basophilic granulocytes [12], increase of basophils and their bone marrow precursors in experimental basophilia [1, 16], and proliferation of the basophilic cell strain in myeloproliferative disorders.

Such observations are sufficient evidence for the bone marrow origin of blood basophils. However, it is not yet known for certain which bone marrow cell ultimately gives rise to basophils. Most authors assume a promyelocytic origin [3, 11, 12, 16]. In contrast, UNDERHILL [17] claims that basophils are a separate cell line with a so-called basophiloblast as stem cell.

In the present work special procedures have been applied in order to demonstrate azurophilic granules as well as metachromatic substances within the same cells. Our aim was to find out whether bone marrow cells disclosing both properties could be detected. If this should be the case, such cells could readily be regarded as proof of the promyelocytic origin of basophils.

Material and Methods

Demonstration of metachromatic granules Air dried normal bone marrow smears were fixed in a 0.1% solution of N-cetyl pyridinium-chloride in methanol formalin for 5 min. Thereafter the smears were stained in a 0.1% solution of toluidine blue in 0.1 M Michaelis buffer, pH 4.0, for 30 min. Then the preparations were differentiated in 0.1 M Michaelis buffer, pH 4.0, for 15 min, and dehydrated twice in isopropanol for 10 sec/each.

Cells with metachromatic granules were photographed and their position was marked. The toluidine blue stain was removed by 0.1 M Michaelis buffer, pH 4.8 and the slides were dehydrated with isopropanol.

Demonstration of promyelocytic (azurophilic) granules In a second step the smears were subjected to the naphthol AS D chloroacetate (NASDCI) esterase reaction [7]. 1 drop of 4% pararosanilin in 2 N HCl was mixed with 1 drop of 4% sodium nitrite in distilled water. After 60 sec 30 ml of 0.1 M Michaelis buffer, pH 7.62 were added. The pH was adjusted to 8.3 with 2 N HCl. 10 mg of naphthol AS D chloroacetate dissolved in 1 ml of N,N dimethylformamide, were added. The mixture was filtered. The smears were incubated for 30 min at room temperature, rinsed in tap water, counterstained with hemalum and mounted in glycerine jelly. Result: nuclei blue, neutrophilic and especially promyelocytic granules red.

Those previously marked cells with positive metachromasia were studied for NASDCI esterase activity and photographed again. In this way, a total of 265 metachromatic basophilic cells was examined using 30 bone marrow smears from 10 different healthy subjects.

Results

As shown previously [14] a broad spectrum of metachromatic substances can be stained by toluidine blue at pH 4.0. This allows a selective demonstration of mature as well as immature basophils.

Mature basophils displayed a strong reddish violet metachromasia of their granules, which were roundish, distinctively delimited and evenly distributed. The nuclei stained faintly blue. With increasing immaturity of the basophils the distribution of metachromatic granules became more irregular and their number decreased. Often they appeared as coarse or thread like structures.

Mature basophilic granulocytes were NASDCI esterase negative. Within the cytoplasm there were well defined empty vacuoles instead of specific granules as a result of their high solubility (fig 1h). However basophilic band forms (fig 1g) displayed a weak but unambiguous NASDCI esterase

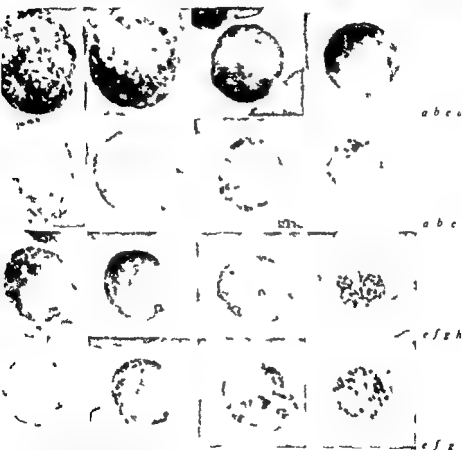


Fig 1 Cells of the basophilic granulocytes subjected to the naphthol AS-D chloroacetate esterase reaction (a-h) after the toluidine blue stain (i-l) $\times 1400$

a b c d

e

f

g

h

i

j

k

l

m

n

top of the cells the toluidine blue metachromatic structures particularly in figures i e e f f and g g

activity The reaction product was diffusely distributed within the intervacular spaces of the cytoplasm Sometimes granular depositions could be seen

Basophilic metamyelocytes (fig 1e, f) presented a *distinct, slightly granular NASDCI-esterase reaction* Myelocytes (fig 1c, d) showed strong promyelocytes (fig 1a, b) very strong enzymatic activity The most immature metachromatic progenitors contained similar NASDCI esterase activities in non specific promyelocytes

The cytotopographical localization of metachromasia and NASDCI-esterase positivity very often revealed a close correspondence This was particularly true for cells already containing well developed metachromatic granules but still possessing a fairly strong NASDCI esterase activity not exceeding that of non specific promyelocytes (fig 1c, d, f)

In general, a definite increase in metachromasia was observed in the course of maturation However, no strict proportionality between the quantity of metachromatic granulation and classical morphological criteria of the different maturation stages could be observed In contrast, the NASDCI esterase activity diminished very constantly, being completely absent in segmented basophils In other words, the enzyme activity closely corresponded to the various developmental stages of the basophils This could be objectivated by cytophotometrical investigations [15]

Discussion

It is generally claimed that basophilic granulocytes develop from non specific promyelocytes [11, 12, 16] Transitions between promyelocytes and specific granulated myelocytes have been described in order to support this view These transitional forms were assumed to be detectable by Pappenheim's stain According to the various descriptions such transitional cells gradually develop specific granules, at the same time losing the promyelocytic granulation

However, a critical evaluation of such descriptions gives rise to considerable doubts about this widely accepted view First of all, it is not clear from the literature how the authors managed to make a clear distinction between promyelocytic and early metachromatic granules in panoptic stains

Secondly, it is quite impossible to demonstrate metachromatic granules adequately by means of panoptic stains, because basophilic granules are highly soluble in aqueous media Accordingly, their preservation requires special procedures [13] In addition, the more immature the cells are, the sparser and the more labile is the metachromatic granulation As aqueous

solutions are used with panoptic stains and as the fixation does not suffice for the preservation of cellular glucomucanes considerable portions of the metachromatic granules are extracted and hence cannot longer be detected. This can easily be shown with any conventionally stained blood smear, in which most of the basophils show more or less empty cytoplasmatic vacuoles which are elated metachromatic granules.

Third, in panoptic preparations promyelocytic granules stain reddish to violet and thus take on a colour very similar to that of the metachromatic granules. Furthermore the cytoplasm of immature cells is strongly basophilic, which further complicates the recognition of single granules. Therefore, in agreement with LEVY [10] we think a reliable distinction between promyelocytic and basophilic granules is not possible by means of panoptic stains. This is particularly true for the identification of the sparse metachromatic granules in early basophilic precursors. So transitions between promyelocytes and early basophils cannot be identified with certainty in panoptic stains.

This shows that the derivation of basophils from promyelocytes had apparently been established by analogy with the development of the neutrophilic cell strain but is not based on unequivocal experimental findings.

The uncertainty of a conventional cytological derivation of the blood basophils is well illustrated by the theories of an experienced cytologist as LEVY [17]. LEVY regards the basophilic cell strain as independent and clearly separate from other granulocytic cell lines. He believes basophils to develop from a so-called basophiloblast. He denies that transitional cells between promyelocytes and basophilic progenitors are demonstrable in panoptic preparations. This is certainly correct as far as Pappenheim's stain is concerned but does not rule out the possibility that some such cells may in fact exist when it being demonstrable by simple stains.

In contrast to panoptic stains the NASDCl-esterase method and the toluidine blue stain reveal objective and reproducible criteria for the recognition of promyelocytic and metachromatic granules respectively. By successive application of these procedures we succeeded in demonstrating all developmental stages from non-specific promyelocytes with strong activities of NASDCl-esterase down to mature segmented basophils without any activity of this enzyme (fig. 1). This is absolute proof of the view that basophilic granulocytes like monocytes [17], eosinophils [8, 9] and neutrophils originate from non-specific promyelocytes.

Another result of our studies concerns the mode of basophilic granule formation. Some authors claim that newly formed specific granules replace azurophilic granules, as the latter gradually disappear during the maturation

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Ultrastructural Localization of Peroxidase Activity in Human Basophil Leukocytes¹

G A ACKERMAN and M A CLARK

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Abstract The cytoplasmic granules of the human basophil leukocyte have been shown to exhibit peroxidase activity both at the light and electron microscopic levels when exposed to the 3,3'-diaminobenzidine tetrahydrochloride peroxidase procedure. Reactivity is diminished when exogenous peroxide is omitted from the incubation medium and is completely abolished when catalase is included in the peroxide-free medium.

Key Words

Basophil leukocytes
Electron microscopy
Peroxidase reaction

Basophil leukocytes have been regarded as being devoid of peroxidase activity [1, 5, 7, 9, 11] although several older studies [4, 6, 8, 10] have reported the presence of this enzyme within the cytoplasmic granules of these cells. It is the purpose of this light and electron microscopic study to provide evidence indicating the localization of peroxidase activity in the granules of basophilic leukocytes of normal human bone marrow using the 3,3'-diaminobenzidine tetrahydrochloride technique.

Materials and Methods

Normal human bone marrow obtained from healthy medical students was processed for the ultrastructural localization of peroxidase activity using the 3,3'-diaminobenzidine tetrahydrochloride (DAB) peroxidase technique as detailed by ACKERMAN and CLARK [2]. Thin sections were examined unstained or stained only with lead citrate or with the uranyl acetate lead citrate sequence. Controls consisted of the elimination of hydrogen peroxide or DAB from the incubation medium or the addition of catalase (3000 BU) to the peroxide-free medium. In addition, dried films of normal human blood were fixed in formalin vapor for 5 min and

¹ Supported by Grant No. AM HE 12084-12 from the National Institutes of Health Bethesda Md.



Fig 1 Peroxidase reactivity is evident in the cytoplasmic granules of the neutrophil (N) and basophil (B) Blood Peroxidase reaction with no counterstain. $\times 1,500$

Fig 2 Peroxidase reactivity is confined to the primary (p) granules of the neutrophil (N) and to the granules (b) of the basophil leukocyte (B) The reactivity of these cytoplasmic granules is greatly masked by the metallic counterstains Bone marrow Peroxidase reaction counterstained with lead citrate and uranyl acetate $\times 12,500$

stained lightly with 0.5% aqueous toluidine blue. The metachromatic basophils were marked and the slides placed in the peroxidase medium for 10 min and the marked basophils examined for peroxidase activity. Fixed films were also checked for peroxidase activity without the intervening staining with toluidine blue.

Results

Basophils identified in fixed films with toluidine blue and subsequently exposed to the peroxidase reaction exhibit distinct staining of their cytoplasmic granules (fig 1). Prior staining of the preparation did not alter the peroxidase reactivity of any of the cellular elements including the basophil leukocyte. At the ultrastructural level, basophil granules are peroxidase-positive, variable in size and contour and frequently exhibit evidence of partial solubilization (fig 2). Most of the basophil granules are stippled in appearance and are larger in size than either the peroxidase-positive primary (azurophil) or peroxidase-negative secondary (specific) granules of the neutrophils. The marked variation in basophil granule size, absence of a central crystalloid and relative sparsity of the granules within the basophil help to distinguish the mature basophil from the mature eosinophil or eosinophilic myelocyte. Counterstaining of peroxidase preparations with lead citrate and uranyl acetate (fig 2) and, to a lesser extent, with lead citrate alone (fig 3) masks the visualization of much of the granule peroxidase reaction product due to the metallophilia of the basophil granules (and granules in other leukocytes). In uncounterstained preparations, strong but variable peroxidase reactivity is evident in the cytoplasmic granules of the mature basophils (fig 4). Moderate granule reactivity also can be demonstrated in these cells in the absence of exogenous peroxide (fig 5). Catalase completely inhibits peroxidase activity in the basophil as well as in other leukocytes and their developmental forms. Immature basophil leukocytes in normal human bone marrow are uncommon but cells identified as basophil myelocytes exhibit reactive cytoplasmic granules, cisternal components (Golgi and rough endoplasmic reticulum) and formative granules.

Discussion

The conflicting reports concerning the presence of peroxidase in the basophil granules may be attributed to the substrate and fixation procedures employed. The cytoplasmic granules of the human basophil leuko-

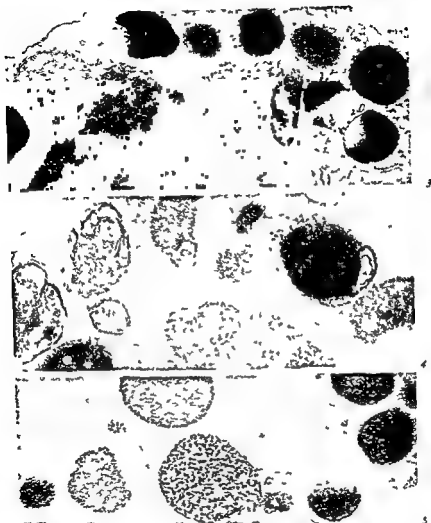


Fig 3 Variations in granular structure and peroxidase reactivity are evident in this mature basophil. Counterstaining of preparation has partially masked the extent of peroxidase reactivity. Bone marrow. Peroxidase reaction counterstained with lead citrate only. $\times 27,500$

Fig 4 Note the strong but variable peroxidase reactivity of basophil granules. Bone marrow. Peroxidase reaction. No counterstain. $\times 35,000$

Fig 5 Basophil granule reactivity is decreased but not prevented in the absence of exogenous peroxide in the incubation medium. Bone marrow. Peroxidase reaction without hydrogen peroxide. No counterstain. $\times 33,000$

cyte yield a positive reaction with the DAB peroxidase technique now commonly being employed in ultrastructural studies of various tissues. The distinction between basophils, eosinophils, neutrophils and particularly their developmental forms cannot be made simply on the basis of the presence or absence of peroxidase reactivity when the DAB peroxidase procedure is utilized since basophilic myelocytes in peroxidase preparations as well as other forms of immature leukocytes (neutrophilic promyelocytes, eosinophilic promyelocytes and myelocytes and promonocytes) involved in the synthesis of peroxidase containing granules exhibit peroxidase activity in their cisternal compartments (rough endoplasmic reticulum, nuclear envelope and Golgi complex) during the period of active granulogenesis [2, 3].

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Pentose-Phosphate Pathway of Erythrocytes in Metabolic Diseases

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Abstract The transketolase activity (TKA) of red cells was determined in 443 subjects: 286 control subjects, 48 diabetics, 29 patients with hyperthyroidism, 8 with hypothyroidism, 11 chronic alcoholics, 25 patients with sideropenia, and 14 with dementia senilis. TKA levels significantly lower than those of the control series were recorded in chronic alcoholics and patients with dementia senilis. The cause of this finding is discussed.

Key Words

Alcoholism
Erythrocyte metabolism
Pentose phosphate shunt
Transketolase

In rat liver, high protein, high fructose, and a starvation-re-feed regimen all produced increases in pentose phosphate metabolizing enzyme activity. Hydrocortisone or cortisone also caused increases, and administration of thyroxine had a negligible effect on the activity of this system. A fast of several days, diabetes, adrenalectomy, and hypophysectomy caused decreases in liver pentose phosphate metabolizing enzyme activity [4]. Thyroxine administration did not affect the transketolase of red cells in the rat [3].

Slightly contradictory views have been published concerning the action of metabolism-affecting enzymes on the pentose phosphate pathway of human red cells. However, it is known that the thyroid hormones usually accelerate oxygen consumption by red cells [2]. Results obtained on red cell hemolysates revealed [1] that hyperthyroidism did not affect transketolase activity. A similar result was recorded in another study of a rather small material [9].

On the other hand [8], apparently the transketolase activity of the blood in thyrotoxicosis may be lower than in healthy human individuals. Reduced glutathione content was observed in red blood cells in hyperthyroidism [12].

¹ Grant from The National Research Council for Medical Sciences, Finland.

It was concluded that the blocking of the pentose-phosphate shunt in hyperthyroidism may be caused by the decrease in reduced glutathione content. NECHELES and BEUTLER [11] assumed the activation of the hexose-mono-phosphate shunt by triiodothyronine *in vitro*.

The present study sought to investigate the effect of certain metabolic diseases and disorders on the pentose-phosphate pathway of human red cells by determining the transketolase activity (TKA) of isolated red cells.

Material and Methods

The series consisted of 443 subjects. Their characteristics are given in table I, which carries the same group numbers as figure 1.

1 *Controls* (286) Some of these subjects were in the hospital for a general medical examination and/or cardiovascular observation. All were in an excellent physical condition and had a good nutritional status.

2 *Diabetes mellitus* (48) All the subjects had had diabetes for several years and had taken either insulin or oral anti-diabetic drugs. They were in the hospital for balancing the disease. Some suffered from certain complications of the disease.

3-4 *Hyper- and hypothyroidism* (37) In both these groups of diseases the clinical symptoms had been clear. The disease had been diagnosed in clinical conditions by determining the basal metabolic rate, serum protein bound iodine, and serum cholesterol on a minimum of 2 occasions. All patients had undergone a radioiodine test. Specific treatment after diagnosis had led to an adequate result.

5 *Chronic alcoholism* (29) All patients were in the hospital for alcohol intoxication of varying degree. Their medical histories revealed alcoholism for several years. All showed signs of neuritis in the extremities.

Table I Characteristics of groups 1-7

No	Group	n	Age, years ±SD	Weight, kg ±SD	Hb g/100 ml ±SD
1	Controls	286	63.8 ± 22.3	69.9 ± 14.8	13.6 ± 1.8
2	Diabetes mellitus	48	62.3 ± 24.3	61.4 ± 18.9	13.5 ± 1.4
3	Hyperthyroidism	29	49.5 ± 12.2	65.3 ± 9.3	13.4 ± 1.4
4	Hypothyroidism	8	51.6 ± 13.3	65.3 ± 17.2	12.0 ± 1.4
5	Chronic alcoholism	29	49.5 ± 12.2	65.3 ± 9.3	13.4 ± 1.4
6	Sideropenia	25	57.0 ± 17.8	65.6 ± 10.3	7.9 ± 2.2
7	Dementia senilis	14	80.8 ± 10.8	49.8 ± 14.5	13.2 ± 1.4

SD = Standard deviation

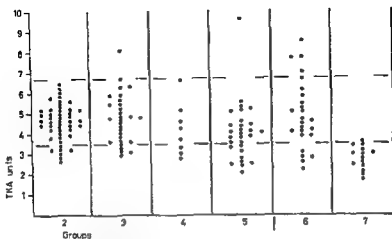


Fig. 1 The TKA of red cells (2) diabetics (4.6 ± 0.8 U) (3) hyperthyroids (4.7 ± 1.2 U) (4) hypothyroids (4.2 ± 1.3 U), (5) chronic alcoholics (4.0 ± 1.4 U), (6) sideropenics (4.7 ± 1.6), (7) patients with dementia senilis (2.8 ± 0.6 U). The dotted lines indicate the limits of normal variation range. The mean \pm SD for controls is 4.8 ± 0.7 U.

6 Sideropenia (25) No disease other than iron deficiency anaemia could be shown in the patients. Iron deficiency was verified by bone marrow examination, serum iron determination and total iron binding capacity determination. The patients had reduced levels of haemoglobin in the blood (table 1).

7 Dementia senilis (14) These geriatric patients had long required institutional care, they were completely desorientated and had no conversational contacts. On a special psychiatric examination the patients' condition was classified as dementia resulting primarily from reduced cerebral blood circulation. They had had no strokes or other distinctly demonstrable diseases apart from those of bones and joints.

All blood samples were taken from venous blood, prepared and stored deep frozen at -20°C .

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fidence limit is $\pm 20\%$.

The individual results for each group are shown in figure 1, in which the horizontal dotted lines indicate the maximum and minimum limits of the individual values in control series.

Results

Figure 1 reveals that all groups studied contained values below and/or above the variations of the control group. Particularly low values were

recorded in the group of chronic alcoholics and in the dementia senilis patients. Among the former, the TKA values of the red cells were below the control variation in one of every 3 patients and in the latter group in 13 of the 14 patients. Statistically the 2 groups also differed significantly from the control group. No such difference was recorded for the other groups studied.

Discussion

The main result of these studies is that chronic alcoholics and dementia senilis patients showed reduced TKA values in red cells as a sign of inactivation in the pentose-phosphate pathway.

The low TKA values in alcoholics need a more detailed etiological analysis. It should be noted in this context, however, that red cell TKA determinations are at present widely used to diagnose thiamine deficiency. It has been shown that fasting or reduced nutrition in a few weeks may reduce the red cell TKA [7]. The diet of alcoholics is known to be unbalanced and often defective for long periods and for these reasons they may be deficient in thiamine. On the other hand it has been shown [5] that experimental liver damage reduced the TKA of red cells. The explanation offered is that the damaged liver is incapable of producing sufficient quantities of the transketolase co-factor [6]. In alcoholics both these factors are realistic facts: they may have a nutritional thiamine deficiency, and their liver is often damaged by alcohol. All patients showed signs of neuritis although only one-third showed TKA values definitely below the variation range of control subjects. Results similar to the present findings have been reported by ALBARIAN and DREYFUS [1] who studied the beri-beri heart disease of alcoholics and by FENELLY *et al* [6] who examined cirrhotic alcoholics.

WOLFE *et al* [13] reported on a series of patients with Wernicke's encephalopathy who were found to have a deficient transketolation in the pentose-phosphate shunt. A parallel finding was recorded in the present patients with dementia senilis. It is presumable that this metabolic change is due to nutritional factors. These patients are incapable of taking care of themselves, especially as regards their food. Furthermore before dementia develops, the aged may have lived on an unbalanced diet, often exclusively composed of carbohydrates. The low average body weight of this group (table I) suggests malnutrition. Studies should be continued by

the experimental administration of thiamine to these patients and investigating its action on their psychic and physical condition

Some earlier authors have claimed that TKA in the red cells falls in hyperthyroidism [8]. The present study did not corroborate this finding, and the red cell TKA in both hyper- and hypothyroidism was approximately equal to the values obtained on the control series. In diabetes, both complicated and non-complicated, the red cell TKA was at a normal level.

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Red-Cell Catalase and the Production of Methaemoglobin, Heinz Bodies and Changes in Osmotic Fragility due to Drugs

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Abstract Heinz bodies were produced *in vitro* in normal human red cells by incubation with ascorbic acid, menadione, sodium azide, primaquine diphosphate, acetyl phenylhydrazine, potassium chlorate, sodium nitrite and *p*-phenetidine. All compounds causing Heinz body formation also produced methaemoglobin and most led to reduction in catalase activity; sodium nitrite and *p*-phenetidine caused no inhibition of catalase. Catalase inhibition was not found with any of the drugs studied which did not produce Heinz bodies. Catalase inhibition appeared to be partly responsible for Heinz body formation by ascorbic acid and menadione, but not for the effects of the other drugs studied. The results of this study support the view that glutathione peroxidase is the most important factor in protecting haemoglobin from the action of oxidant drugs.

Key Words

Catalase
Erythrocyte metabolism
Glutathione peroxidase
Haemolysis by drugs
Haemoglobin oxidation
Heinz bodies
Methaemoglobin

Hydrogen peroxide is generated within normal red cells after the addition of primaquine, phenylhydrazine, menadione and other oxidant drugs [9]. Catalase was at one time regarded as the main protection against the oxidative destruction of haemoglobin by hydrogen peroxide, formed during intracellular metabolism [13]. Glutathione peroxidase has been shown to protect haemoglobin from oxidation by hydrogen peroxide produced within the red cell under physiological conditions by the action of ascorbic acid [18, 26], and COHEN and HOCHSTETTER [8] considered that glutathione peroxidase was the primary agent for the elimination of hydrogen peroxide in red cells. There is evidence that both catalase and glutathione peroxidase may play a part in destroying hydrogen peroxide, and the relative importance of each of the two enzymes is not yet clear.

One manifestation of the toxic effect of oxidant drugs on red cells is the appearance of intracellular inclusions (Heinz bodies), which represent the end product of the degradation of haemoglobin. BRENNER and ALLISON [6] pointed out that a common property of toxic substances producing Heinz bodies was catalase inhibition, and they suggested that the accumulation of hydrogen peroxide within the red cell caused the formation of Heinz bodies.

The present study was undertaken to study the correlation between catalase inhibition and the formation of Heinz bodies and methaemoglobin in normal human red cells incubated *in vitro* with various drugs and chemical substances. In addition the effects of these compounds on osmotic fragility and on the activities of erythrocyte glutathione peroxidase and glucose 6 phosphate dehydrogenase have also been studied.

Methods and Materials

Incubation of red cells. Venous blood was taken in a dry sterile syringe and defibrinated in a sterilised Erlenmeyer flask containing glass beads. Using aseptic technique an aliquot of blood was removed for measurement of the haemoglobin concentration. A further volume of blood was incubated in a sterile solution of 0.85 percent sodium chloride (containing 0.01M phosphate buffer pH 7.0) in a glass container so that the final haemoglobin concentration in the incubation mixture of 25 ml was 2.0 g/100 ml. All drugs added were dissolved in buffered saline (except sulphadiazine and nitrofurantoin) and a suitable volume of this solution was added in making up the incubation mixture so that the desired final concentration of the drug was obtained. Sulphadiazine and nitrofurantoin did not dissolve in buffered saline in the concentration required but were soluble in plasma; with these drugs only the incubation mixture was made up by diluting the defibrinated blood with plasma.

The cell suspensions were incubated in a waterbath at 37°C for 16 h, after which the suspensions were centrifuged and the supernatant solutions were removed. The haemoglobin concentration of the supernatant was measured and the percentage haemolysis which had occurred during incubation was calculated. The red cells were washed twice with 15 vol of cold isotonic saline and aliquots of the washed cell suspension were used for the measurement of Heinz bodies, osmotic fragility and methaemoglobin, and for the preparation of haemolysates for the assay of catalase, glucose-6-phosphate dehydrogenase and glutathione peroxidase.

Heinz bodies. To 0.5 ml of red cell suspension in a test tube was added 1 ml of a filtered solution of crystal violet (40 mg/100 ml) and the tube was placed in a water bath at 37°C for 30–60 min. The mixture was then centrifuged, the supernatant discarded and smears of the cells washed in buffered saline as described by

Heinz bodies As the spontaneous formation of Heinz bodies in incubated red cells is accelerated with increasing dilution of the cells [5] experiments were performed similar to those described above except that the haemoglobin concentration of the final incubation mixture was 40 g/100 ml instead of 20 g. Control samples without added drugs showed Heinz bodies in not more than 1% of cells. This difference in the dilution of the cells in the incubation mixture did not appreciably influence the effect of the added compounds on Heinz body formation.

Osmotic fragility of the incubated red cells was studied by a standard technique [7] haemolysis being measured by the optical density of cyanmethaemoglobin at 540 nm.

Methaemoglobin was estimated by measuring the ratio of optical densities at 576 and 590 nm [17]. Control cells incubated without additives showed less than 10% of methaemoglobin.

Catalase was assayed by a titrimetric method using potassium permanganate as previously described [33]. **Glutathione peroxidase** was measured by the method of PAGLIA and VALENTINE [29] and the activity was expressed in enzyme units, as defined by these workers. **Glucose-6 phosphate dehydrogenase** was measured by the method described by TARLOV and KELLERMEYER [32]. The enzyme activity was expressed as the change in optical density/min/mg of haemoglobin.

Glucose concentration was estimated by the adaptation of the Somogyi method described by NELSON [27].

The following compounds were obtained from the Sigma (London) Chemical Company Ltd: catalase (beef liver) C/30, glutathione reduced (crystalline), glutathione reductase (yeast) type III, TPN and TPNH, menadione, sodium bisulphite, primaquine diphosphate, D-glucose 6 phosphate disodium salt, Phenacetin, acetanilide, paracetamol and sulphanilamide were obtained from Hopkins and Williams, *p*-phenetidine hydrochloride from Kodak Ltd, nitrofurantoin (Furandantin) was kindly donated by Smith, Kline and French Laboratories; all other compounds used were of reagent grade.

Results

Heinz bodies were present in 50–100% of red cells after incubation with the substances shown in table 1. These compounds were studied over a range of concentrations from 0.1 to 100 mM and results are shown only for the lowest concentration with which the average Heinz body count was greater than 50%. At these concentrations methaemoglobin formation always occurred, but there was no close correlation between the percentage of cells showing Heinz bodies and the amount of methaemoglobin (table 1). Similar high percentages of Heinz bodies were associated with 95% methaemoglobin (sodium nitrite) and with 30% (acetyl phenylhydrazine). With the concentrations of drugs shown in table 1, the haemolysis occurring during incubation was always equivalent to less



Fig 1 Heinz body formation *a* Red cells after incubation with acetylphenyl hydrazine (10 mM) showing large Heinz bodies 1-2 per cell *b* Red cells after incubation with ascorbic acid (100 mM) showing multiple small Heinz bodies *c* Red cells after incubation with menadione (0.1 mM) showing multiple Heinz bodies $\times 440$

Table 1 Heinz bodies methaemoglobin and catalase activity of normal red cells after incubation for 16 h at 37°C with various compounds

Test substance and concentration (mM) in incubation mixture	Percentage of red cells showing one or more Heinz bodies	Methaemoglobin %	Catalase % of activity of incubated control cells
Ascorbic acid (10.0)	92.2±11.2	42.8±9.7	12.7±4.9
Menadione (0.1)	89.2±15.4	71.4±10.7	34.5±6.2
Sodium azide (1.0)	66.9±20.9	35.1±12.6	55.0±4.5
Primaquine diphosphate (1.0)	81.6±27.7	63.7±10.8	64.6±5.7
Acetyl phenylhydrazine (1.0)	97.4±3.3	29.7±6.2	77.8±2.0
Potassium chlorate (5.0)	52.2±18.7	75.7±11.4	81.2±8.2
Sodium nitrite (10.0)	88.5±9.9	95.1±5.4	100.0
p-Phenetidine (10.0)	50.8±29.2	82.6±12.2	100.0

The values shown are the means and standard deviations of the results from 10 experiments

than 3% of the total haemoglobin concentration of the incubated red cells

Inhibition of catalase activity, of varying degrees, occurred with all the substances listed in table 1 except sodium nitrite and p-phenetidine. There was no close correlation between the degree of catalase inhibition and the percentages of Heinz bodies or of methaemoglobin. Marked inhibition of catalase was produced by menadione and ascorbic acid but much less inhibition occurred with primaquine and acetyl phenylhydrazine, at concentrations producing a similar percentage of red cells with Heinz bodies (table 1).

With some compounds the appearance of the Heinz bodies was characteristic. Thus the large, round bodies, usually 1 or 2 per cell, produced by acetyl phenylhydrazine (fig. 1a), contrasted strongly with the multiple inclusion bodies produced by the other drugs – small with primaquine or ascorbic acid (fig. 1b) and usually slightly larger with menadione (fig. 1c). The effects of potassium chlorate differed from that of the other substances studied in that at low concentrations (<5 mM), 5–25% of the cells showed Heinz bodies, but at higher concentrations, 5.0 mM and greater, diffuse blue staining of almost all cells occurred with crystal violet, although discrete darker inclusions, which were counted as Heinz bodies could be seen in the majority of the cells.

No significant production of Heinz bodies, or of methaemoglobin, and no inhibition of catalase occurred on incubation of normal red cells with the following compounds phenacetin (5 mM), acetanilide (10 mM), paracetamol (10 mM), sulphanilamide (5 mM), sulphadimidine (5 mM), nitrofurantoin (5 mM), sodium nitrate (10 mM) and sodium sulphite (10 mM)

The effects of 3-amino-1,2,4-triazole (AT) and of ethanol The addition of AT (2 mM) to the incubation mixtures containing menadione, ascorbic acid or primaquine resulted in complete inhibition of catalase and a significant increase in the formation of Heinz bodies and of methaemoglobin (table II) When AT, in concentration of 2 mM was incubated with red cells without the addition of other drugs, it did not produce Heinz bodies, methaemoglobin or inhibition of catalase With acetyl phenylhydrazine, sodium azide, sodium nitrite, potassium chlorate and *p* phenetidine, the addition of AT did not influence significantly the effects of these compounds in producing Heinz bodies or methaemoglobin, or in inhibiting the activity of catalase

Table II The effects of 3-amino-1,2,4-triazole (AT) (2 mM) and of ethanol (100 mM) on the formation of Heinz bodies and methaemoglobin during incubation of red cells with ascorbic acid, menadione and primaquine diphosphate

Test substance and concentration (mM) in incubation mixture	Heinz bodies % cells			Methaemoglobin %		
	without AT	with AT	significance	without AT	with AT	significance
Ascorbic acid (1.0)	63.6	89.2	0.001 < p < 0.01	42.8	55.0	p < 0.001
Ascorbic acid (10.0)	40.1	72.9	p < 0.001	17.8	30.2	p < 0.001
Menadione (0.01)	81.6	100.0	0.01 < p < 0.05	63.7	76.8	p < 0.001

Effect of ethanol

Test substance and concentration (mM) in incubation mixture	Heinz bodies % cells			Methaemoglobin %		
	without ethanol	with ethanol	significance	without ethanol	with ethanol	significance
Ascorbic acid (1.0)	63.6	47.2	0.001 < p < 0.01	42.8	25.2	p < 0.001
Ascorbic acid (10.0)	89.2	87.1	p > 0.1	71.4	52.9	0.001 < p < 0.01
Primaquine (1.0)	81.6	85.6	p > 0.1	63.7	68.8	p > 0.1

The mean values from 10 experiments are shown and also the significances of the differences between results with and without AT or ethanol

When ethanol (100 m*M*) was added to the incubation mixtures containing menadione, ascorbic acid or primaquine, the catalase inhibition by these compounds was completely prevented. Ethanol reduced the formation of Heinz bodies and of methaemoglobin by ascorbic acid, and reduced methaemoglobin with menadione (table II), it had no significant effect on the degree of these changes produced by primaquine. With all the other drugs studied, ethanol did not influence the effect of the drug on catalase activity, or on the formation of Heinz bodies or methaemoglobin.

The effect of glucose The addition of glucose (20 m*M*) to the incubation mixture greatly reduced the formation of Heinz bodies and of methaemoglobin with all the compounds which produced these effects, except with sodium azide (table III). The protective effect of glucose in preventing Heinz bodies was least apparent with menadione, and in preventing methaemoglobin, was least with sodium nitrite (table III).

Osmotic fragility Increased osmotic fragility, outside the range of results of 50 experiments with incubated normal cells, was found with primaquine (1 m*M*), ascorbic acid (10 m*M*), *p*-phenetidine (10 m*M*), potassium chlorate (5 m*M*) and sodium sulphite (10 m*M*). With menadione, osmotic fragility was normal after incubation with the compound in concentration of 0.1 m*M*, but abnormal with 1.0 m*M* (fig. 2). The osmotic fragility was within the normal range after incubation with all the other

TABLE III The effect of glucose (20 m*M*) on the formation of Heinz bodies and methaemoglobin during incubation of red cells with various compounds

Substance and concentration (m <i>M</i>) in incubation mixture	Heinz bodies % cells			Methaemoglobin %		
	without glucose	with glucose	significance	without glucose	with glucose	significance
Ascorbic acid (10.0)	92.2	4.0	$p < 0.001$	42.8	24.0	$p < 0.001$
Menadione (0.1)	89.2	45.2	$p < 0.001$	71.4	32.4	$p < 0.001$
Sodium azide (1.0)	66.9	55.9	$p > 0.1$	35.1	41.1	$p > 0.1$
Primaquine diphosphate (1.0)	81.6	0.7	$p < 0.001$	63.7	35.2	$p < 0.001$
2,2'-Bipyridyl phenylhydrazine (1.0)	97.4	12.8	$p < 0.001$	29.7	21.9	$p < 0.01$
Potassium chlorate (5.0)	52.2	26.5	$0.01 < p < 0.001$	75.7	53.3	$p < 0.001$
Sodium nitrite (10.0)	88.5	5.0	$p < 0.001$	95.1	84.1	$0.01 < p < 0.001$
<i>p</i> -Phenetidine (10.0)	50.8	5.6	$p < 0.001$	82.6	71.9	$p < 0.001$

The mean values from 10 experiments are shown and also the significances of the differences between pair of results with and without glucose.

compounds studied With menadione ascorbic acid and primaquine, addition of glucose (20 mM), AT (2 mM) or ethanol (100 mM) to the incubation mixture did not produce any marked or consistent change in the effect of the compound on osmotic fragility

Glutathione peroxidase and G6PD Menadione consistently reduced the activity of both enzymes In 6 experiments the glutathione peroxidase activity of incubated control cells was 6.4 ± 1.3 enzyme units (mean \pm SD), whereas after incubation with menadione (0.1 mM), the activity was 1.9 ± 0.9 enzyme units ($0.01 > p > 0.001$)

In 11 experiments, the glucose 6 phosphate dehydrogenase activity of control incubated cells was expressed by a mean change in optical density of $13.7 \pm 3.1 \times 10^{-3}$ /min/mg of haemoglobin, whereas the mean activity after incubation with menadione (0.1 mM) was $5.2 \pm 2.8 \times 10^{-3}$ /min/mg haemoglobin ($p < 0.001$)

After incubation of red cells with primaquine, ascorbic acid, acetyl phenylhydrazine, sodium nitrite, phenacetin, paracetamol, sulphadimidine and nitrofurantoin in the concentrations indicated above, no consistent or

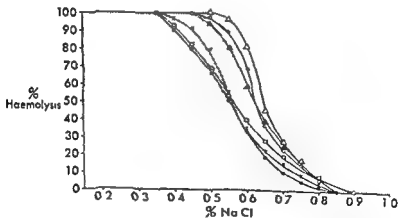


Fig 2 Osmotic fragility after incubation of normal red cells with various compounds For each substance the values shown are the mean results from 5 experiments The shaded area indicates the range of results of 50 experiments with normal cells incubated in the same way, without additional substances ○ Ascorbic acid 10 mM □ *p*-phenetidine 10 mM △ sodium sulphite 10 mM × primaquine 1 mM ● menadione 1 mM ▲ potassium chlorate 5 mM

significant changes occurred in the activities of glutathione peroxidase and glucose-6-phosphate dehydrogenase

Glucose concentration in incubation mixture In 30 experiments, the glucose concentration of the control cell suspension was measured at the beginning and end of incubation. There was a mean reduction in concentration of 1.67 mM, the final concentration at the end of incubation being 0.42 ± 0.19 mM. In experiments in which glucose was added to the incubation mixture in an initial concentration of 20 mM, the concentration of glucose at the end of incubation was 14.0 ± 0.88 mM.

Discussion

Drug induced haemolysis commonly results from a direct effect of the compound on red-cell metabolism, less frequently, haemolysis is associated with the production of an immune reaction. Many substances, which have a direct toxic action on red cells, lead to destructive oxidation of haemoglobin and probably also of the cell membrane [11]. The compounds are either oxidising agents, such as potassium chlorate, or they lead to the formation of hydrogen peroxide or other oxidising agents within the red cells. COHEN and HOCHSTEIN [9] demonstrated that hydrogen peroxide was generated during the incubation of red cells with various haemolytic agents, including primaquine, phenylhydrazine and menadione. Oxidation of haemoglobin by hydrogen peroxide leads to the formation of methaemoglobin and subsequently to the irreversible precipitation of the denatured haemoglobin as Heinz bodies [20]. Most of the evidence has indicated that methaemoglobin is an essential preliminary step in the oxidative destruction of haemoglobin, including the formation of Heinz bodies [15, 16, 20], but BEUTLER and BALUDA [4] suggested that the formation of methaemoglobin and Heinz bodies are parallel processes, and that methaemoglobin is not necessarily an essential precursor of the precipitation of degraded haemoglobin. In the present study there is a close association between Heinz bodies and methaemoglobin in that no compound produced a significant degree of one of these changes without also producing the other, however, as in the study of MILLER and SMITH [25], there was no quantitative correlation between the percentages of methaemoglobin and of Heinz bodies produced. The size of the Heinz bodies varied with the compound used, this is presumed to be related to the speed of formation, as ALLEN and JANDL [1] showed that drugs with more rapid effects produced smaller Heinz bodies.

BRENNER and ALLISON [6] suggested that inhibition of catalase might be important in the oxidative damage by chemical substances, as compounds capable of producing Heinz bodies were also catalase inhibitors. The present study has shown that, whereas most compounds which produced marked Heinz body and methaemoglobin formation also inhibited catalase, this was not invariable, and no inhibition of catalase was observed with sodium nitrite or *p*-phenetidine. There was no close correlation between the degree of inhibition of catalase and the magnitude of the effects of oxidation of haemoglobin. The compound 3-amino-1,2,4 triazole (AT) produces rapid irreversible inhibition of catalase only in the presence of a low concentration of hydrogen peroxide [22], this reaction has been used to demonstrate the presence of low levels of hydrogen peroxide [9, 34]. Ethanol prevents the formation of the inactive catalase hydrogen peroxide complex II [23]. The generation of hydrogen peroxide during incubation of red cells with ascorbic acid, menadione and primaquine was confirmed by the experiments with AT and ethanol. With ascorbic acid and menadione, the increased catalase inhibition produced by the addition of AT was accompanied by increased Heinz bodies and methaemoglobin, ethanol reduced these effects with ascorbic acid, and reduced methaemoglobin production with menadione. These results suggest that catalase inhibition may have played some part in the oxidation of haemoglobin by ascorbic acid and menadione. With primaquine, although AT and ethanol produced marked changes in catalase activity, they did not influence the oxidative effects. With the other compounds tested there was no evidence that catalase inhibition was directly contributing to the formation of Heinz bodies or methaemoglobin. The lack of close correlation between alterations in osmotic fragility and the production of Heinz bodies, methaemoglobin and catalase inhibition is in keeping with previous observations [16-25]. Although changes in the red-cell membrane, resulting in increased osmotic fragility and haemolysis occur as a result of oxidant drug action, these changes are not necessarily the direct result of oxidation of haemoglobin. Some substances appear to produce relatively greater effects on the membrane than on haemoglobin and *vice versa*.

There has been much interest in the relative importance of catalase and of glutathione peroxidase in protecting the normal red cell from the destructive effects of hydrogen peroxide [8, 28]. Glutathione peroxidase was shown to protect haemoglobin from oxidation by ascorbic acid [18, 26]. COHEN and HOCHSTEIN [8] concluded that glutathione peroxidase was the major pathway of hydrogen peroxide metabolism in red

cells. Other workers have reported evidence suggesting that both catalase and glutathione peroxidase are normally active in protecting the red cell from hydrogen peroxide [19, 30]. Glutathione peroxidase requires a constant supply of NADPH, which, in turn, depends on the activity of the hexose monophosphate shunt [8]. During the incubation of red cells without added glucose, depletion of red-cell glucose occurs so that the protective effect of glutathione peroxidase is lost [26]. In the present study, with all the compounds studied except sodium azide, methaemoglobin and Heinz body formation were greatly reduced by the addition of glucose to the incubation mixture. The protective effect of glucose, more apparent on Heinz bodies than on methaemoglobin, is in keeping with previous reports [15, 16, 26].

Menadione produced marked Heinz body formation on incubation with red cells from normal adults. Hyperbilirubinemia has resulted from high doses of vitamin K analogues in newborn infants [2, 24]. In the newborn, the increased susceptibility of the red cells to oxidative damage [5] is, at least partly, attributable to the low levels of activity of catalase and of glutathione peroxidase [14]. The potential haemolytic effect of menadione in such infants is probably increased by the inhibitory effect of the compound, shown *in vitro*, on catalase and glutathione peroxidase, menadione also reduced the action of glucose-6 phosphate dehydrogenase, as previously reported [12].

Methaemoglobin and Heinz bodies occurred after incubation with *p*-phenetidine, no such effects were observed with phenacetin, paracetamol or acetanilide. Phenacetin, in clinical use, may cause methaemoglobinaemia and haemolysis and it is likely that these effects are due to one of its metabolites, possibly *p*-phenetidine. In relation to the clinical toxicity of phenacetin, it is of interest that there appear to be genetically controlled variations in the metabolism of the drug with consequent differences in the proportions of the various metabolites produced [31].

With sodium nitrite, no inhibition of catalase, and no change in osmotic fragility occurred despite high percentages of Heinz bodies and of methaemoglobin. BEUTLER [3] noted that sodium nitrite, although causing marked methaemoglobinaemia, did not cause haemolysis of either normal or G6PD deficient red cells. The failure of nitrite to produce haemolysis *in vivo*, despite its ability to produce hydrogen peroxide and to oxidise haemoglobin [10], may be explained by the reaction of nitrite with catalase-hydrogen peroxide complex I [21] thus detoxifying hydrogen peroxide as well as generating it.

Acknowledgements We are very grateful to Mr R FAWKES and Mr J CORN HILL for photographic assistance, this research programme has been supported by a grant from the Scottish Hospital Endowments Research Trust

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Erythropoietic Cell Proliferation in Different Clinical States of β -Thalassaemia¹

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Abstract Erythropoietic cell proliferation of 6 cases of different clinical states of β -thalassaemia (thal) was studied by a combined method using Feulgen-cytophotometry and autoradiography after *in vitro* labelling with ^3H -TdR. An abnormal distribution of the DNA content and ^3H -TdR incorporation was found, which was more pronounced in the one patient with thal major than in the 3 patients with thal minor and was not provable in the subclinical form (mutina), indicating a direct relationship to the ineffective erythropoiesis and the states of anaemia in this disease. This proliferation disturbance consisted of an accumulation of cells in G_1 , a decreased proportion of cells in S and a decreased S/ G_1 ratio, and was limited to the early polychromatic cell compartment. In the basophilic erythroblasts, a decreased proportion of cells in G_1 was observed revealing a shortening of the G_1 period in this cell compartment.

Key Words

Autoradiography
Cytophotometry
DNA synthesis
Erythropoiesis
Thalassaemia

In β thalassaemia (thal) apart from the basic defect of decreased β -chain synthesis, the erythroid hyperplasia of the bone marrow, rapid iron turnover and low iron incorporation provide evidence of ineffective erythropoiesis [8]. From comparative ferrokinetic studies in cases of thal with different severity of clinical symptoms, correlations between the degree of anaemia and ineffective erythropoiesis have been suggested [4]. Recently, in homozygous thal a severely disturbed proliferation of early polychromatic erythroblasts could be demonstrated by a combined Feulgen photometric and auto-

¹ Supported by Deutsche Forschungsgemeinschaft

Concerning this problem, the present study was undertaken with special regard to erythropoietic cell proliferation in cases of heterozygous β -thal different from haematological data. The results give good evidence that in comparison with β -thal major, the degree of proliferative disturbance of the early polychromatic cell compartment decreases with the severity of haematological symptoms.

Material and Methods

Patients Six patients have been investigated. The diagnosis of β -thal was established by haemoglobin analysis (starch block electrophoresis, alkali denaturation, acid elution technique) and other haematological findings (table 1). Classification of heterozygous β -thal cases into minor and minima forms was followed by clinical and haematological symptoms.

General procedure The marrow was aspirated into a syringe containing 0.5 ml EDTA solution (1% Na-EDTA in 0.7% NaCl), and incubated for 1 h with ^3H -TdR (concentration 2 $\mu\text{Ci/ml}$, specific activity 2 Ci/mu) at room temperature. Smears were made from the marrow spicules and stained with the May-Grünwald-Giemsa stain. The individual cells were marked and photographed for subsequent localization, for consecutive cytophotometry and autoradiography. Thereafter, Pappenheim stain was leached out by treatment with 50% ethanol, and the smears were restained by the Feulgen method applying pararosanilin for Schiff's reagent. For the cytophotometric determination of the DNA content a MPV-cytophotometer (Leitz, FRG) was used. For the determination of the nuclear size the nuclear diameters were evaluated. Autoradiograms of the Feulgen stained smears were made by the dipping film technique. The technical details of cytophotometry and autoradiography have been described previously [6, 7].



Results

Evaluation of the Diploid Standard and of the Different Stages of the Cell Cycle

The combined application of the cytophotometric determination of the DNA content and autoradiographic labelling with ^3H -TdR *in vitro* allows to distinguish the following cell groups representing the different stages of the cell cycle:

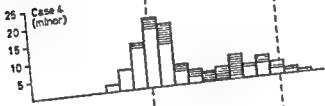
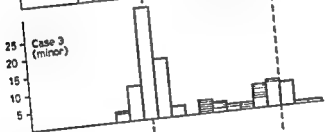
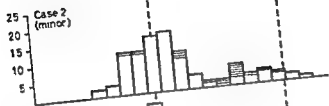
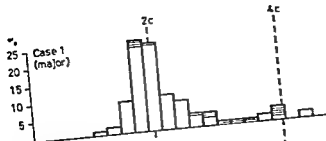
- a) Cells within the diploid range showing no autoradiographic labelling (G_1)
- b) Unlabelled cells within the tetraploid range (G_2)
- c) The cells labelled with ^3H -TdR, which mostly show a DNA content between diploid and tetraploid values and according to the distributional

Table 1 Main laboratory data of 6 patients with β -thalassaemia

Diagnosis	No	Case	Hb, g/100 ml	RBC, 10 ⁶ /μl	Hk, %	MCH, pg	MCV, μm ³	Reti- c- ulocytes %/100	Total bili- rubin, mg%	Hapto- globin, mg%	Serum- Fe, μg/100 ml	B O
Homozygous thal. (major)	1	S A., female born 1969	6.9	2.92	19.0	24.0	66	26	2.1	86.0	0	88 69 1
Heterozygous thal. (minor)	2	M P., male, born 1937	10.3	4.72	33.0	22.0	70	29	7.6	1.5	0.42	0 136 38 1
	3	T G., male, born 1921	10.5	4.29	29.5	24.1	69	82	4.5	8.6	3.40	0 131 70 1
	4	A L., female, born 1930	8.2	4.30	28.0	18.0	65	27	6.7	0.6	2.06	10.0 131 35 1
Heterozygous thal. (minor)	5	M H., female, born 1937	12.9	5.50	38.0	24.0	69	5	5.2	0.5	0.43	- 152 06 1
	6	P D., male, born 1965	12.2	6.16	38.2	19.1	62	12	5.5	2.8	0.72	68.0 129 09 1

E G = Erythro-granulopoietic ratio (normal range 0.2-1.0-8.1)

E G = Erythro-granulopoietic ratio (normal range 0.2-0.8)



've DNA content (AU) and ^3H -TdR -labelling (shaded areas) in the early
 oblasts of patients with β -thalassaemia. Semilogarithmic scale. Abs-
 t in arb
 dar
 units; ordinata percentage of cells 2c=diploid

error of the absorption photometry sometimes are localized within the diploid or tetraploid range (S)

d) Unlabelled cells with a DNA content between diploid and tetraploid level which cannot be attributed to one of the resting periods (U = unrecognizable)

For evaluation of the diploid standard (2c) the DNA values of all basophilic and early polychromatic cells in G_1 have been averaged. The mean tetraploid value (4c) was calculated from 2c

DNA Content of Erythroblasts in Thalassaemia

In all 6 cases of *thal* the DNA content of the *basophilic erythroblasts* was distributed from the diploid to the tetraploid level indicating the presence of a complete cell cycle in this cell type. In cases 1, 2, 4 and 6 cells were slightly accumulated at the tetraploid level, in case 5 a distinct accumulation of diploid cells was observed. No significant deviation from the pattern of normal erythropoiesis was found. The late polychromatic erythroblasts were diploid.

As seen in figure 1, in the *early polychromatic erythroblasts* an accumulation within the diploid range was observed in homozygous *thal* (major) and heterozygous *thal* with clinical symptoms (minor). In case 1 only few cells were hyperdiploid and tetraploid. In the cases 2-4 a somewhat higher proportion of cells between 2c and 4c and within the tetraploid range was observed. In heterozygous *thal* without clinical symptoms (minima) (case 5-6) an appreciable proportion of cells between 2c and 4c was observed. In agreement with this observation, no distinct accumulation of diploid and tetraploid cells (except at 4c in case 6) was found.

DNA Synthesis Stages of Erythroblasts in Thalassaemia

The percentage distribution of erythroblasts of the 6 cases of *thal* in the different stages of the cell cycle is given in table II. In the *basophilic erythroblasts*, the cells in S did not show a remarkable difference according to the different clinical state of *thal*. In comparison to normal values (table II), the proportion of cells in G_1 is somewhat decreased and the proportion of cells in G_2 is distinctly increased. In case 5 (minima) these differences have not been observed.

In the *early polychromatic erythroblasts* striking differences are present between the cases studied. As compared to the values found in normal erythropoiesis, the proportion of cells in S is evidently low in homozygous *thal* and somewhat higher in heterozygous *thal* with clinical symptoms and

Table II Percentage distribution of erythroblasts in the different

Diagnosis	No	Basophilic erythroblasts		E1		U	S/G ₁
		n	G ₁	S	G ₂		
Thalassaemia major	1	100	20.0	64.0	13.0	3.0	4.9
Thalassaemia minor	2	148	18.2	55.4	23.0	3.4	2.4
Thalassaemia minor	3	107	16.8	61.7	20.6	0.9	3.0
Thalassaemia minor	4	126	11.9	76.2	11.1	0.8	6.9
Thalassaemia minima	5	88	38.6	48.9	9.1	3.4	5.4
Thalassaemia minima	6	108	12.0	68.5	17.6	1.9	3.9
Thalassaemia minima			27.9	63.6	7.9	0.6	8.1
Normal (4 cases)		mean		24.5-37.3	51.3-70.1	4.8-10.7	0.1-1.1
		range					5.0-12.2

n = Number of cells, U = unrecognizable cells (see text)

within the normal range in heterozygous thal without clinical symptoms. On the other hand a high proportion of cells in G₁ was observed in homozygous thal, a somewhat lower proportion in heterozygous forms showing anaemia. In heterozygous thal without clinical symptoms no difference was found from normal erythropoiesis. According to this the S/G₂ ratio increased from a very low level in homozygous thal to the normal range in heterozygous thal without symptoms. In the late polychromatic erythroblasts most of the cells were found in G₁ (or in G₀) as seen in normal erythropoiesis.

Discussion

Ineffective erythropoiesis is the most important mechanism responsible for the anaemia and increased total erythropoietic activity in thalassaemia. It cannot readily be explained by the disturbance of haemoglobin synthesis *per se*, but points to a connected abnormality of erythroblastic proliferation.

DNA synthesis stages (G_1 , S, G_2) in patients with β -thalassaemia

Early polychromatic erythroblasts, E_4						Late polychromatic erythroblasts, E_5				
n	G_1	S	G_2	U	S/ G_2	n	G_1	S	G_2	U
186	82.3	9.7	5.3	2.7	1.8	42	97.6	0	0	2.4
171	72.5	13.5	12.9	1.1	1.0	64	100.0	0	0	0
122	64.8	14.8	18.9	1.5	0.8	33	100.0	0	0	0
117	49.5	33.6	13.1	3.8	2.6	112	99.1	0	0	0.9
111	30.1	54.8	15.1	0.0	3.6	106	93.4	6.6	0	0
103	17.5	67.0	13.6	1.9	4.9	138	95.3	1.5	0	3.2
	18.7	65.3	15.2	0.8	4.3		96.0	4.0		
	8.4	54.9	4.9	0	3.9		94.6	0		
	29.9	74.8	26.3	1.4	14.0		100.0	5.9		

and/or maturation leading to intramedullary cell death. Abnormal patterns of proliferation in homozygous β -thal have been observed by WICKRAMASINGHE *et al* [11] and KESSELIAS *et al* [3] using the combination of Feulgen photometry and ^3H TdR autoradiography. In the present study, the erythropoietic cell proliferation of one patient with homozygous and 5 patients with heterozygous β -thal of different clinical intensity (table I) was investigated by this technique. It was one of the objectives of this investigation to decide whether the changes found by the above mentioned authors were quantitatively connected with the degree of ineffective erythropoiesis present in different clinical forms of β -thal.

An abnormal distribution of the DNA content and ^3H -TdR incorporation was found in the 4 patients with haematologically symptomatic β -thal (major and minor), but not in the subclinical forms (minima). Within the symptomatic group, the deviation from normal was qualitatively alike but more pronounced in the one patient with β -thal major. Because comparable ferrokinetic studies of MALAMOS *et al* [4] in β -thal major, minor and minima clearly demonstrated a correlation between the degree of erythropoietic inefficiency

and the intensity of the clinical symptoms, it is suggested that the disturbance of erythroblast proliferation observed by WICKRAMASINGHE *et al* [11] and ourselves is directly related to the ineffective erythropoiesis and the anaemia in this disease

As in the work of WICKRAMASINGHE *et al* [11], the significant abnormalities in our patients were limited to the early polychromatic cell compartment. They consisted of an accumulation of cells in G_1 , a decreased proportion of cells in S and consequently a decreased S/ G_2 ratio (fig 1, table II). These changes may be explained by the lack of induction of DNA synthesis in a large fraction of the early polychromatic cells. Probably these nonproliferative early polychromatic erythroblasts do not extrude the nucleus and enter the peripheral circulation, but are destroyed within the marrow. The small number of late polychromatic normoblasts seen in the marrow of symptomatic thal may be the result of the small fraction of early polychromatic cells entering the cell cycle or of maturation without additional mitosis. Of course, the methods used in this investigation are not suitable for explaining the connection between the underlying defect of β chain synthesis and the premature shutoff of DNA synthesis observed.

A different explanation of the ineffective erythropoiesis in thal has been given by KESSE-ELIAS *et al* [3]. These authors found an increased proportion of unlabelled cells between 2c and 4c (U-cells) and suggested an arrest of DNA synthesis within S-phase analogous to the pattern observed in megaloblastic anaemia [5, 10, 12], in sideroblastic anaemia [9] and in dyserythropoietic anaemia type I [7]. In the present study the percentage of the U-cells was only slightly increased and failed to show any correlation to the severity of the disease. Therefore, it seems unlikely that the mechanism suggested by KESSE-ELIAS *et al* [3] is responsible for the ineffective erythropoiesis. The discrepancy of the results of these authors on one side, and the results of WICKRAMASINGHE *et al* [11] and ourselves on the other may be explained either by differences in technique or by a secondarily folate deficiency possibly present in the homozygous patients of KESSE-ELIAS *et al* [3].

In the basophilic erythroblasts an increased proliferation activity has been demonstrated in cell culture studies using colchicine [1]. Special attention to the basophilic cells was given in an autoradiographic *in vitro* study using the double labelling technique with 3H - and ^{14}C -thymidine [2]. In this study a shortening of the DNA synthesis time from the normal average of 17.9 to 10.0 h and a shortening of the generation time from 23.0 to 13.4 h was observed in 9 cases of thalassaemia major. The results of the present study give no information about the time parameters of the mitotic cycle. However,

the decreased proportion of cells in G_1 (table II) together with these data reveal a shortening of the G_1 period in this cell compartment, which can be understood as a compensatory mechanism of the proliferation disturbance described in the early polychromatic cell compartment

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The Incidence of β -Thalassemia and Abnormal Hemoglobins in Turkey

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Abstract The present study has been carried out to estimate the gene frequency of thalassemia and abnormal hemoglobins in a random Turkish population. A total of 1,000 blood samples have been studied. Fifteen of 900 adults had elevated Hb A₂ concentrations. 0.60% of the population studied had an electrophoretically demonstrable hemoglobin abnormality, 3 Hb AS, 1 Hb AE, 1 Hb AD and one Hb A₁'s ('split' A₁). Signs of a thalassemia were found in one case. This study indicates that the incidence of β -thalassemia trait in Turkish population as 1.66%.

Key Words

Abnormal haemoglobins
Haemoglobin A₂
Thalassemia
Turkey

Various forms of thalassemia and abnormal hemoglobins have been observed in Turkey for many years [1, 3-5, 7, 9, 11, 13, 14, 17, 21, 22]. It is known that β -thalassemia gene has a widespread distribution in the mediterranean countries [16, 29]. The incidence studies of thalassemia from Italy, Greece, Israel, Spain have been reported [10, 20, 23, 24, 26]. Although Eti-Turks, a small community living in southern part of Turkey, have been repeatedly studied in regard to abnormal hemoglobins and thalassemia [2, 3, 9], there has been no survey conducted to determine the incidence of β -thalassemia and hemoglobinopathies in a random Turkish population. Therefore the present study has been carried out to estimate the gene frequency of β -thalassemia and abnormal hemoglobins in Turkey.

The second group comprised 100 cord blood samples taken from City Maternity Hospital Ankara, Turkey. The true origin of the subjects were searched as carefully as possible. This study was based on the measurement of Hb A₂ level without having a prior hematologic screening due to the fact that the surveys utilizing osmotic fragility and hematological data alone may not be too accurate [29].

Cyanmethemoglobin hemolysates were subjected to starch block electrophoresis according to the method of KUNKEL and WALLENTUS [18] with slight modification. One minute alkali denaturation test was performed by the method of SINGER [27]. Agar gel electrophoresis was done by the method of ROBINSON [25] to differentiate Hb S from Hb D. Starch-gel electrophoresis was also performed to separate minor hemoglobin fractions whenever necessary [28]. Complete hematologic studies were carried out on all subjects found to have elevated Hb A₂ values. Erythrocytes glucose-6-phosphate dehydrogenase was tested qualitatively in 300 cases.

Results

Fifteen of the 900 adults tested had elevated Hb A₂ concentration, an incidence of 1.66%. The Hb A₂ level ranged from 3.8% to 6.8% with a mean value of 4.6%. Six individuals or 0.60% of the population studied had an electrophoretically demonstrable hemoglobin abnormality. In addition to the 15 with elevated Hb A₂, 3 had sickle cell trait (0.30%), one had heterozygous Hb D (0.10%) and one had Hb A₂ (0.10%) (table I). Hematological values in cases with elevated Hb A₂ level were within the range seen in heterozygous β -thalassemia (table II). The hemoglobin concentration ranged from 10.4 to 15.9 g%, red blood cell counts from 4.40 to 5.71 million/mm³, hematocrite from 31 to 53%, MCV from 69 to 98 μ m³, MCHC from 23 to 32%. The reticulocyte counts were all within the normal limits. The serum iron levels ranged from 98 to 285 μ g%. The total iron binding capacity varied from 275 to 443 μ g%. The osmotic fragility curves revealed increased red cell resis-

Table I Distribution of hemoglobin types in 1,000 Turkish subjects

Hemoglobin type	No	%
AS	3	0.3
AE	1	0.1
AD	1	0.1
A ₂ (B ₂)	1	0.1
AA	994	99.4
Total	1 000	100

The Incidence of β -Thalassemia and Abnormal Hemoglobins in Turkey

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Medical School of Ankara University, Ankara

Abstract The present study has been carried out to estimate the gene frequency of thalassemia and abnormal hemoglobins in a random Turkish population. A total of 1,000 blood samples have been studied. Fifteen of 900 adults had elevated Hb A₂ concentrations. 0.60% of the population studied had an electrophoretically demonstrable hemoglobin abnormality: 3 Hb AS, 1 Hb AE, 1 Hb AD and one Hb A₁² ('split' A₂). Signs of a thalassemia were found in one case. This study indicates that the incidence of β -thalassemia trait in Turkish population is 1.66%.

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Material and Methods

A total of 1,000 blood samples from 2 sources have been studied. Firstly 900 samples were taken from healthy subjects in the Turkish Army and the Medical School. These subjects were selected to provide a better representation of different regions of the country.

The second group comprised 100 cord blood samples taken from City Maternity Hospital Ankara, Turkey. The true origin of the subjects were searched as carefully as possible. This study was based on the measurement of Hb A₂ level without having a prior hematologic screening due to the fact that the surveys utilizing osmotic fragility and hematological data alone may not be too accurate [29].

Cyanmethemoglobin hemolysates were subjected to starch block electrophoresis according to the method of KUNKEL and WALLFUS [18] with slight modification. One minute alkali denaturation test was performed by the method of SINGER [27]. Agar gel electrophoresis was done by the method of ROBINSON [25] to differentiate Hb S from Hb D. Starch-gel electrophoresis was also performed to separate minor hemoglobin fractions whenever necessary [28]. Complete hematologic studies were carried out on all subjects found to have elevated Hb A₂ values. Erythrocytes glucose-6-phosphate dehydrogenase was tested qualitatively in 300 cases.

Results

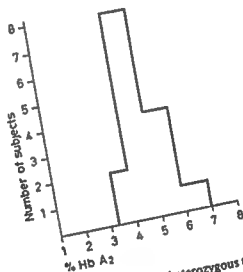
Fifteen of the 900 adults tested had elevated Hb A₂ concentration, an incidence of 1.66%. The Hb A₂ level ranged from 3.1% to 6.8% with a mean value of 4.6%. Six individuals or 0.60% of the population studied had an electrophoretically demonstrable hemoglobin abnormality. In addition to the 15 with elevated Hb A₂, 3 had sickle cell trait (0.30%), one had heterozygous Hb D (0.10%) and one had Hb A- (0.10%) (table I). Hematological values in cases with elevated Hb A₂ level were within the range seen in heterozygous β -thalassemia (table II). The hemoglobin concentration ranged from 10.4 to 15.9 g%, red blood cell counts from 4.40 to 5.71 million/mm³, hematocrite from 31 to 53%, MCV from 69 to 98 μ m³, MCHC from 23 to 32%. The reticulocyte counts were all within the normal limits. The serum iron levels ranged from 98 to 285 μ g%. The total iron binding capacity varied from 275 to 443 μ g%. The osmotic fragility curves revealed increased red cell resis-

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AA	994	99.4
Total	1,000	100

Table II Hematological findings in 15 cases of heterozygous β -thalassaemia

	Hb, g %	RBC $\times 10^6$	Htc, %	MCHC %	Red-cell morphology	Serum iron $\mu\text{g} \%$	Hb A ₂ %	Hb F, %	Osmotic fragility
Range	10.4-15.9	4.40-5.71	31-53	23-32	Hypochromia and basophilic stippling very common	98-285	3.8-6.8	0.6-5.9	%0.5-%0.1
Mean	12.8	5.36	41.8	29.1		173.7	4.63	2.0	Decreased

Fig 1 Distribution of Hb A₂ in heterozygous thalassemia

tance in all cases of heterozygous thalassemia. The stained blood films were remarkable for hypochromia and basophilic stippling in the majority of the cases. Target cells and aniso-poikilocytosis were less apparent. The fetal hemoglobin value was elevated to 5.9% in only one case. The distribution of Hb A₂ values is shown in figure 1. No glucose-6-phosphate deficiency was found among the studied cases.

Discussion

The present study indicates that elevated Hb A₂ values are usually diagnostic of thalassemia trait because the cases with an elevated Hb A₂ in the

survey had hematologic values commonly found in thalassemia trait. Since the initial observation of KUNKEL and WALLFRIUS [29] this finding has become the main diagnostic feature in heterozygous β thalassemia, although the absence of a high value does not exclude thalassemia. Therefore, our figure of 1.66% incidence of elevated Hb A₂ levels in Turkish population may be taken as an indication of the incidence of heterozygous thalassemia associated with an increase in the Hb A₂ fraction but does not necessarily reflect the true incidence of thalassemia in Turkey.

The incidence of 1.66% of β -thalassemia appears to be closer to that found in northern and central Italy [29] and Spain [23] but lower than that found in Greece (10, 20). Incidence of thalassemia has shown a marked variation in Israel, the highest being found in the tribe of Indian origin [24, 29].

The distribution of 15 cases of heterozygous thalassemia according to their origin is shown in figure 2. In this survey it was not possible to demonstrate any area of very high incidence in Turkey. However, if the geographical regions are taken into consideration, the incidence seems to be higher in the southern part of Turkey. It is of interest that all cases with an elevated Hb A₂ showed the hematological abnormalities commonly seen in thalassemia traits, however an elevation of Hb F was found in only one case. This may partly be due to the fact that Hb F was determined in a relatively low number of

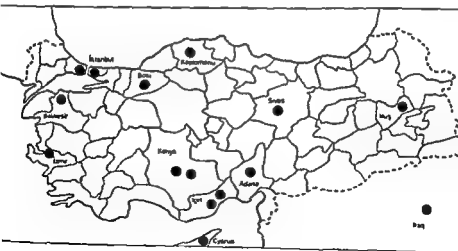


Fig. 2

blood samples (374 cases) Nevertheless an elevation of Hb F does not seem to be a consistent feature in thalassemia trait in this series This finding seems to be quite different from that of Greek and Spanish studies in which the cases of β thalassemia with an increase of Hb F only have been reported [10, 20, 23] Therefore elevation of Hb A₂ appears to be a more characteristic finding for β -thalassemia in Turkish sample, although AKSOY has reported a few families with heterozygous thalassemia having high Hb F levels from this country [6]

The most prominent morphological findings were hypochromia and basophilic stippling The latter was previously shown to be very common in the Italian and Greek thalassemia heterozygotes [29] A recent report from Spain indicates that basophilic stippling was the least frequent feature among the Spanish subjects [23]

From the data presented herewith, the following calculations can be made Approximately 16 665 in 1 million of the Turkish population can be expected to have heterozygous β thalassemia and 71 in 1 million homozygous thalassemia

It should be noted that cases without an elevation of Hb A₂ or Hb F could not be detected by the present study However the incidence of this type of thalassemia is likely to be low, since both parents of the children with thalassemia major diagnosed in this department (to be reported separately) usually had an elevation of Hb A₂ indicating that type II thalassemia with normal Hb A₂ is not commonly seen in Turkey [12]

No relationship between malaria and thalassemia could be found in this study Firstly, malaria is practically eradicated in Turkey, secondly the frequency of thalassemia was not significantly high in any area in the present survey

During this study one case with low Hb A₂ was found He had hematological findings compatible with thalassemia trait However his Hb A₂ level was repeatedly low and Hb F was normal Although no Hb H or Bart's could be detected, we incline to consider this boy having a thalassemia since the family studies on our previous cases of Hb H-disease in Turkey revealed low Hb A₂ values in several members of the same family Furthermore a few red cells containing inclusion bodies may be unnoticed However, the possibility of δ -thalassemia cannot be ruled out completely The rest of the family was not available for further study

Among the 6 electrophoretically demonstrable hemoglobin abnormalities 3 had Hb AS, one had Hb AE, one had Hb AD and one had Hb A'₂ ('split' A₂) The incidence of 0.60% of abnormal hemoglobin is lower than

that found in Eti-Turks. It should be stressed that these cases were not related to Eti-Turks. Indeed a recent survey done on this community revealed that the incidence of Hb S and E were 13.8% and 2.43% respectively contrary to β -thalassaemia which was considerably low with an incidence of 0.81% [9].

One case had Hb A₂' (split A₂) or Hb B₂ as previously called Hb A₂' a variant of Hb A₂ was originally described in American negroes particularly in the state of Georgia [15] The person in our study with this abnormality had no negroid features and she was the first case found among Turks, the same anomaly was only reported once previously from Turkey in a person of Greek extraction [8] A recent survey in Indonesia has shown an incidence of about 3% of Hb A₂' [19] The abnormality thus has a world wide occurrence in many ethnic groups The occurrence of A₂' in Turks probably indicates a blood admixture with the people of South East Asia

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Megaloblastic Anemia due to Folic Acid Deficiency in a Young Woman on Oral Contraceptives

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Clinique médicale thérapeutique universitaire (Prof. R. S. MACH)
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Abstract Case report of a young woman aged 27, suffering from severe megaloblastic anemia due to folic acid deficiency. The factor responsible for this deficiency seems to be the regular use of oral contraceptives for 3 years (Noracycline 22®). Recent investigation evidenced the existence of selective malabsorption of food folates due to the action of hormonal contraceptives on the intestinal conjugase. It can thus be admitted that contraceptive drugs play a prominent part in the genesis of this type of anemia. However, other factors must also be considered, like malnutrition and a masked malabsorption syndrome. The relation with megaloblastic anemia in pregnancy is discussed.

Key Words

Folic acid deficiency
Megaloblastic anemias
Contraceptives and anemia

Among the numerous effects of hormonal contraceptives [2], attention has recently been drawn to their interference with folic acid absorption. Serum folic acid level is depressed in women taking regularly and for more than one year oral contraceptives [4, 9]. A few cases of anemia have even been described in this situation [11], this type of anemia responded either to oral folic acid, or to a simple discontinuation of the contraceptive medication. Hormonal contraceptives seem to have the same action as diphenylhydantoin on the intestinal enzyme responsible for the deconjugation of food folates [1, 12, 14] and thus inhibit their absorption. However, it seems unlikely that this mechanism should be alone responsible for the development of this type of anemia. Other factors, such as a masked malabsorption of different origin, or a malnutrition, should also be considered.

We recently observed a young woman with severe megaloblastic anemia due to folic acid deficiency. In this patient oral contraceptives

played a prominent part. We have tried to outline the possible action of an occult malabsorption syndrome

Case Report

A young woman, aged 27 consults her physician for fatigue, dyspnea on effort and transient malleolar edema. She complains of having lost about 10 kg in 4 months, without any change in appetite. Food intake has always been adequate, but several months ago she developed a distaste for meat. The patient also noticed some degree of hair loss and was recently affected with buccal aphthae. Except for the regular use (during 3 years) of a contraceptive medication (Norethylone 22 mg, each tablet containing Mestranol 0.075 mg and Lynestrol 2.5 mg) she takes no other drugs, and in particular, no anticonvulsants.

Her previous medical history is unremarkable. She had a normal pregnancy 6 years ago and no anemia was reported on that occasion. The patient never had any gastro-intestinal complaints, her stools are normal in frequency and consistence.

Finally the family medical history is negative especially with respect to pernicious anemia, diabetes, vitiligo thyroid gland diseases or malabsorption syndromes.

The medical examination shows a young woman in satisfactory general health condition. She weighs 49 kg for a height of 155 cm. Temperature 37.4°C. Subcutaneous sclerae very pale teguments. The nails are striated and brittle. The hair is scarce with a starting frontal baldness. Blood pressure at 120/70 mm Hg regular pulse at 132/min with an apical systolic murmur of 2/10 intensity. The tongue is well papillated and the buccal mucosa is healthy. The liver shows no enlargement, the spleen is not palpable. No anomaly was observed during the detailed neurologic examination. The eye fundus revealed a small superficial hemorrhage in each eye.

Hemoglobin 4.6 g % and 7.9 g % after transfusion (1 U). Hematocrit 22.5%. Red cells 1 700 000/mm³. MCV 132 μm³. MCH 46 μg. MCHC 35%. Anisocytosis macro-ovalocytosis with a few erythroblasts (fig 1). Reticulocytes 45%. Leucocytes 5 620/mm³ with 53% segmented neutrophils (with several clearly hypersegmented forms) 63% non segmented neutrophils 1% eosinophils 1% basophils, 6% monocytes and 32.5% lymphocytes. Platelets 292 000/mm³.

Bone marrow Erythroid hyperplasia (ME = 11) marked megaloblasticosis and giant metamyelocytes. Normal thrombopoiesis. Sedimentation rate 6/15 mm. Glycemia fasting 0.85 g/l. Bilirubin total 15 mg/l. 1 direct 4.7 mg/l. Serum iron 2.4 mg/l. Iron-binding capacity 3.4 mg/l. Cholesterol 17 g/l. SGO and SGPT transaminases 9 and 7 IU/l. Prothrombin level 100%. Fibrinogen 1.2 g/l. Serum albumin 4.5 g/l. Lactic dehydrogenase (LDH) 633 IU/l. Electrophoresis increase in α-globulins. Determination of immunoglobulins IgG 830 mg% IgA 120 mg% IgM 45 mg%. Calcium 9.1 mg/l. Phosphorus 4.6 mg/l. Carotene 0.55 mg/l (normal = 0.62-3.12 mg/l). Vitamin A 0.46 mg/l (normal = 0.2-0.8 mg/l).



Fig 1 Blood smear (May Grunwald Giemsa) Megalo-ovalocytes and hypersegmented polynuclears ■

Fig 2 Bone marrow smear (May Grunwald Giemsa) Promegaloblast × ■

Induced hyperglycemia after ingestion of 100 g saccharosis 1.32 g/l after 2 h
50 g lactose is followed by a maximum value of 1.14 g/l after 30 min

D-xylose test after ingestion of 25 g urinary excretion of 4.75 g in 5 h

Fat content of stools (on diet containing 100 g fat daily) 0.4 g/24 h

Stool analysis no anomaly, except for the presence of albumins. Absence of blood or parasites bacteriologic analysis negative

Gastrointestinal X rays neither morphologic nor functional anomaly of the small intestine

Biopsy of jejunal mucosa well developed villusities no anomaly of stroma of blood and lymph vessels

Secretine test volume 1.9 ml/kg/80 min (normal = 1.9-7.4) maximum HCO_3^- concentration 84.3 mEq/l (normal = 11-152 mEq/l)

Differential diagnosis of megaloblastic anemias Dosage of serum folic acid 1.4 ng/ml (normal 5-27 ng/ml) Dosage of serum vitamin B_{12} 72 μg /ml (normal 120-470 μg /ml) Schilling test urinary excretion of 21% of the ingested activity in 24 h Kay test (stimulation of gastric secretion by 0.25 mg s.c. Pentagastrin) basal secretion (MBAO) 0.1 mEq HCl (normal = 2+2 mEq) maximal acid output (MAO) 11.4 mEq (normal = 10-17 mEq)

Investigation for autoantibodies against intrinsic factor and stomach parietal cells negative Investigation for LE cells and other autoantibodies negative except for positive thyroidal anti-microsome antibodies at 1/16 (complement fixation)

A therapeutic test (fig 3) with vitamin B_{12} (1 μg /day i.m.) gives no response whereas folic acid (500 mg Folvite/day i.m.) is followed by a typical reticulocyte response The contraceptive medication has not been interrupted during this test

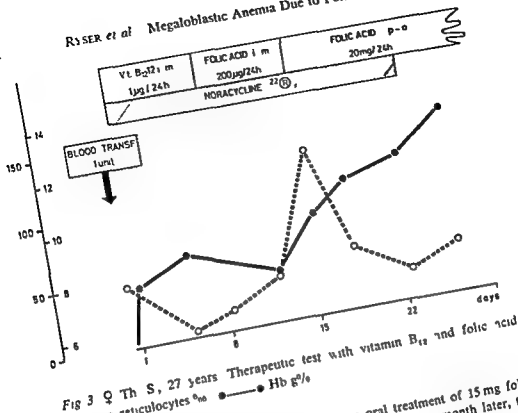


Fig 3 ♀ Th S, 27 years Therapeutic test with vitamin B₁₂ and folic acid
 ○---○ reticulocytes % ●—● Hb g%

Evolution Anemia was completely cured with an oral treatment of 15 mg folic acid without discontinuation of the contraceptive medication. One month later, the values are Hb 14.6 g%, red cells 4,550,000/mm³, leukocytes 8,900/mm³, serum iron 1.25 mg/l and iron binding capacity 3.0 mg/l.

Discussion

A folic acid deficiency is undoubtedly responsible for this type of megaloblastic anemia. Low serum folate level, typical response to the clinical trial, no disturbance of the vitamin B₁₂ metabolism, positive response of anemia to folic acid therapy.

In this patient, none of the main causes of folic acid deficiency [13] were present: her food was adequate and, except for a dislike of meat, her appetite was not reduced. She is neither alcoholic nor epileptic and did not take any drugs which might impair folic acid metabolism. The various tests did not point to a malabsorption syndrome. Consequently, it is tempting to consider the oral contraceptive responsible for this anemia. As a matter of fact, various similar cases have been reported by STREIFF [12] and PATON [5], who observed a good response of the anemia to a daily administration of 250 µg folic acid, or in 2 patients, to a

simple discontinuation of the contraceptive medication. According to SHOJANIA *et al* [8, 9], women taking regularly and for more than 1 year oral contraceptive, have a significantly lower serum folic acid level. However, it must be noted that SPRAY [10] did not confirm these results.

Food folates (polyglutamates) are only absorbed as monoglutamates in the small intestine, after deconjugation by an intestinal enzyme, the Bc-conjugase [1, 7]. Hormonal contraceptive drugs (combined type) seem to be responsible for a selective malabsorption of food folic acid, indeed, STREIFF [11] could evidence that a malabsorption of food folates (polyglutamic folates) exists in these patients, whereas the monoglutamic form of folate is normally absorbed. Inhibition of Bc-conjugase must then be admitted [1]. The same mechanism has been evidenced with diphenyl hydantoin [2, 5, 14].

Folic acid deficiency anemia occurs frequently during the 3rd trimester of pregnancy. This condition has always been explained by increased requirements due to foetal growth [11], however, according to these observations, one must admit that the pathogenesis of this anemia can include some degree of food folate malabsorption.

Since in some pregnant women with megaloblastic anemia a slight malabsorption syndrome has been observed [15], the problem of a pre-existing intestinal disease also exists. But what is the explanation for the rare occurrence of this type of anemia when the use of hormonal contraceptives is so widespread? With SHOJANIA *et al* [9] we must consider that it is very unlikely that a megaloblastic anemia of this type would develop solely from contraceptives but that this may only have been a contributory factor.

Two other factors must be considered in our patient: (1) some degree of malnutrition, at least during the last months before hospital admission, among the women tested by SHOJANIA [9], those who had a poor dietary intake had the lowest serum folic level, (2) an occult malabsorption syndrome. Although most of the tests and the histology of the jejunal mucosa were normal some values were low or borderline, like serum carotene and vitamin A levels, calcemia and curves of induced hyperglycemia.

Further investigation is necessary to determine whether an occult malabsorption syndrome does exist in our patient or in others [5, 9], whether of congenital or acquired type (selective or not), which might explain the importance of the anemia in presence of the contraceptive drug.

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Stable Suspension of Erythrocytes Suitable for Calibration of the Electronic Counters¹

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Abstract A simple method of preparing stable suspensions of normal erythrocytes is described. These suspensions, stable for number, shape and volume without aggregates and easy to use, are available as a standard of reference for the calibration and further controls of the electronic counters.

Key Words
Cell counters
Erythrocyte counting
Stable erythrocytes
Standardization

The problem regarding the availability of corpuscle suspensions stable for number, shape and volume without aggregates and easy to use, is still an open question [1-6, 8]. Apart from many other possible uses, a type of preparation with these characteristics would be of great value as a standard of reference for the calibration and successive daily control of the electronic counters.

In order to obtain a stable standard suspension of particles to be used in the calibration of the electronic counters, we have tested our new fixing solution [9] which was slightly modified during the last 3 years [11].

Material and Methods

The fixative has at present the following composition: crystallized acetic acid, 42.0 mg; sodium sulphate anhydrous, 7.0 g; sodium chloride anhydrous, 9.0 g; distilled water, 1000.0 ml.

After preparation the solution must be carefully filtered. Whole blood is added to the fixing solution in the proportion of 1 to 200. The stock suspension obtained

¹ Supported by a grant from the Consiglio Nazionale delle Ricerche (Special Program TBM).

in this way should be stored in the freezer. This stock suspension is suitable for the erythrocyte optical counts and allows the subsequent preparation of dilutions suitable for every type of electronic counters.

Our stock suspension has been controlled during 2 years for morphologic numerical and volumetric stability of its corpuscles. The cytomorphologic behaviour has been studied with a phase microscope. The numeric stability has been controlled by means of optical and electronic counts carried out by methods reported previously [10]. The study of the particle size distribution in the standard suspension has been carried out by the control of the plateau obtained with electronic counters.

We used the Celloscope 101 Counter and the Biotronics 400 B Counter for both the electronic counts and the study of the plateau (particle size distribution) for both counters (Coulter type) apply principles of electrical gating to the problem of particle counting [7]. The cells suspended in an electrically conducting medium flow through a small aperture. The relatively nonconducting cells cause a voltage drop as they pass through the aperture. The resulting pulses, each of which is proportional to the size of the cell inducing it, are amplified and appear on an oscilloscope. Those exceeding values given by the selected threshold setting are recorded on a decade counter.

When counting RBC we used corresponding threshold settings for both electronic counters which permitted the registration of all pulses corresponding to all erythrocytes whatever their size.

For the study of the particle size distribution of the RBC suspensions we performed successive counts using increasing threshold settings starting from a minimal value which unambiguously records all pulses due to RBC up to values of threshold settings which exclude progressively increasing numbers of RBC from registration. In this way using the numerical values obtained in a given range of threshold settings we were able to obtain a plateau with both fixed and fresh control cells thus evaluating and comparing the particle size distribution. This was possible since 2 corpuscle suspensions having similar plateaus will have an even more similar corpuscle size distribution.

During the first 5 months we controlled our standard suspension by an experimental specific Electronic Analyzer of the volumetric distribution of particles working in connection with the Biotronics 400 B Counter^{*}.

Results

Controls over 2 years performed by phase microscope, confirmed the good morphological preservation of the fixed corpuscles and the absolute absence of stable aggregates (fig 1). During the same time optical and electronic counts proved the numeric constancy of the corpuscles in the corpuscular standard suspension.

* This experimental analyzer has been constructed by Dr M. FRANK, Head of the Electronic Laboratories of the Istituto Superiore di Sanità of Rome.

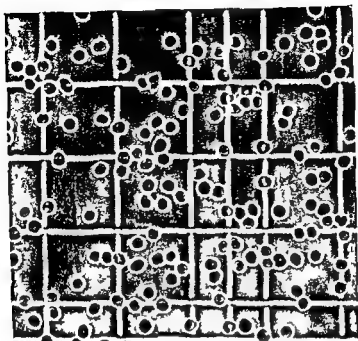


Fig 1 Fixed red cell suspension after 2 years (phase microscope)

The plateau obtained with the electronic counters until 6 months after the preparation of the standard suspension showed results similar to those obtained with normal RBC (fig 2) Only after 12 months the plateau of the fixed corpuscles showed a slight decrease of its width

Moreover also the volumetric distribution of our suspension, studied during 5 months by the electronic analyzer was found to be similar to that of the normal RBC (fig 3)

Discussion

These studies prove the numeric stability of our fixed suspension of erythrocytes Moreover the particle size distribution after 6 months results still similar to that obtained with normal fresh RBC suspensions Because of these characteristics the experimented standard suspension is

TORLONIA/TATA

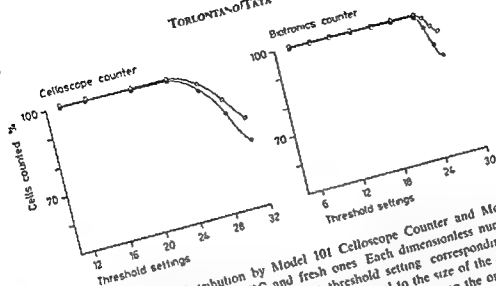


Fig 2 Particle size distribution by Model 101 Celloscope Counter and Model 400/B Counter, comparing fixed RBC and fresh ones. Each dimensionless number on the abscissa indicates a specific instrument threshold setting corresponding to the lowest value of pulses, the size of which is proportional to the size of the corresponding corpuscles, from which the pulses are registered. Numbers on the ordinate indicate the per cent of pulses and thus of the cells present in the suspensions counted and registered at each threshold setting. ○ Fresh RBC, ● fixed RBC after 6 months.

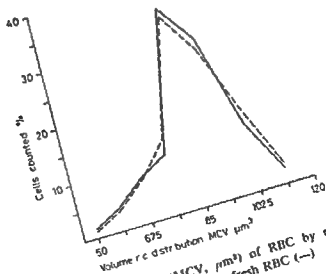


Fig 3 Volumetric distribution (MCV, fm^3) of RBC by electronic analyzer comparing fixed RBC after 5 months (---) and fresh RBC (—).

suitable as a standard of reference for the calibration and further daily controls of the electronic counters

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Kinetics of Erythroid Bone Marrow Cells of Normal and Porphyric Calves *in vitro*¹

W G RUDOLPH and J J KANEKO

Abstract The *in vitro* proliferation and maturation of bone marrow cells in erythropoietic porphyrin of cows was studied using ³H thymidine labeling and autoradiography. Ratios of basophilic normoblast and orthochromatic normoblast were 1:3:8:19 for normal marrow and 1:3:12:32 for porphyric marrow. The turnover times for the basophilic normoblast, late polychromatic normoblast, polychromatic normoblast, late polychromatic normoblast were 10, 17, 31 h in the normals as compared to 12, 16, 26 h in the porphyrics. The cell cycle times and DNA synthetic times were correspondingly similar.

Key Words
Autoradiography
Bone marrow culture
Calve bone marrow
Erythropoietic porphyrin

Erythropoietic porphyrin is a rare congenital disease of man and cattle in which the site of abnormal porphyrin formation is the immature developing erythrocyte. The primary defect in this disease involves overproduction of type I porphyrins due to an enzymatic defect in the conversion of porphobilinogen to uroporphyrinogen [14]. Type I porphyrins cannot be utilized for hemoglobin synthesis and their presence may interfere with the normal process of erythropoiesis or cell survival. Since the most active phase of hemoglobin formation is in the later normoblast stages [13] and these exhibit the highest intensity of porphyrin fluorescence [10], these cells could also be affected with abnormal proliferative or maturative processes. This investigation was designed to study these effects in porphyric bone marrow cells *in vitro*.

¹ This study was supported in part by the University of California University of Chile Co-operative Program and USPHS Grant HE 6678

Table 1 Porphyrin concentrations in blood and urine

		Calf No			
		Normal		Porphyric	
		421	371	2323	2324
RBC	coproporphyrin	Tr	Tr	6.7	38.5
	protoporphyrin	Tr	Tr	186	210
Plasma	coproporphyrin	Tr	Tr	Tr	1.5
	protoporphyrin	Tr	Tr	Tr	Tr
Urine	coproporphyrin	Tr	Tr	625	700
	uroporphyrin	Tr	Tr	480	385

Values are $\mu\text{g}/100\text{ ml}$, Tr = trace amount

Materials and Methods

Two normal calves (421 and 371) and 2 porphyric calves (2323 and 2324) were selected as bone marrow (BM) donors. The porphyric calves were maintained in an enclosed barn and protected from sunlight. Calves 371 and 2324 were used twice as donors. Porphyrin concentrations in these calves are given in table 1.

Bone marrow sampling and incubation procedure. The marrow was withdrawn aseptically by sternal puncture using heparin as anticoagulant. Samples were kept chilled in ice for 1 h and then the incubation procedure was begun. All incubations were carried out aseptically in duplicate at 37°C for 48 h in a carbon dioxide incubator. The incubation mixture was slightly modified from that reported by KRANTZ *et al.* [7]. Incubations were started (0 time) with homologous serum, penicillin and ^3H thymidine² ($1.15\ \mu\text{Ci}/10^7$ nucleated cells) and BVI cells at a concentration of 5 000 to 10 000 nucleated cells mm^3 . After 4 h of labeling time³, NCTC 109 medium at 1.5 times the volume was added. Samples were removed from the flasks at different time intervals and nucleated cell counts were performed on an aliquot. The remaining cells were then washed twice with cold saline and the volume adjusted to 0.2 ml with saline. Smears were then made on $25 \times 75\text{ mm}$ microscope slides and fixed in methyl alcohol.

Autoradiography. Autoradiographs of the smears were made using the Kodak AR 10

² Isotopes

³ Schwarz BioResearch Inc., Orangeburg, NY. SA 3 Ci/ml, diluted with sterile saline solution to contain $200\ \mu\text{Ci}/\text{ml}$.

Results

The number of erythroid BM cells, basophilic normoblasts (Bn), polychromatic normoblasts (Pn) and late polychromatic normoblasts (LPn) gradually decreased in number during the 48 h incubation. The orthochromatic normoblasts (On) gradually increased so that throughout the incubation period the total number of erythroid cells remained constant.

The ratios of Bn, Pn, LPn, and On were 1:3:8:19 in normal BM and 1:3:13:35 in porphyric BM at 1 h of incubation. After 24 h of incubation, morphological alterations such as vacuoles in the cytoplasm were observed in the erythroid cells equally in normal and porphyric BM. The respective ratios at 24 h were 1:4:17:45 and 1:5:17:70. At 48 h, the ratios were 1:4:26:168 and 1:16:20:197, respectively. There was an initial decrease in number of On during the first 6 h of incubation which was then followed by the increase. Chilling is a known means of synchronizing cells in culture and this initial decrease in On was attributed to the resulting alteration in proliferation of the younger precursors.

Grain count per labeled cell The grain count over each labeled cell reached a maximum at 6 h in both experimental groups but remained at a plateau level for 6 h in the normal and for only 3 h in the porphyrics. Marked differences were observed among the different erythroid cells. The youngest cells incorporated the greatest amount of ^3H -thymidine. The ratios of average grain counts of Bn, Pn, LPn and On at the time of maximum incorporation were essentially the same, 4:2:2:1 in the normals and 3:2:1:1 in the porphyrics. The disappearance patterns of label in the Bn, Pn and LPn were also similar in both experimental groups.

Percentage labeled cells The percentage of labeled cells in Bn, Pn, and LPn of both experimental groups were 50, 40 and 30 respectively at 1 h of incubation. At this same time, the percentage of labeled On was only about 5%. In both groups, the percent-labeled cells reached a maximum at 6 h but again, the maximum values were maintained for 6 h in the normal whereas in porphyric BM, it was maintained for only 3 h. The peak On labeling occurred between 24-36 h in both groups and the curves showed a typical relationship where On was the product of its precursor LPn. The rate of increase of labeled On in the porphyrics was 1.14%/h as compared to 1.91%/h in the normals. This would indicate that the transfer of label to On is faster in normal BM as compared to porphyric BM.

Specific activity (SA) of labeled cells The SA of Bn, Pn, and LPn was obtained by multiplying the percent-labeled cells by the mean grain count/

Table II Kinetics of erythroid bone marrow cells

	Labeled cells, %	T_c , h	T_s , h	T_t , h
<i>Normal bone marrow</i>				
Basophilic normoblast	82	7.3	6.0	10.5
Polychromatic normoblast	70	11.5	8.0	16.6
Late polychromatic normoblast	60	21.3	12.8	30.7
<i>Porphyric bone marrow</i>				
Basophilic normoblast	92	8.6	7.9	12.4
Polychromatic normoblast	82	11.2	9.2	16.2
Late polychromatic normoblast	■	17.7	12.0	25.5

labeled cell. The data showed an exponential decrease in SA. From the slopes, k , of the decreasing SA, the cell cycle times (T_c or the half time) and turnover times (T_t) were calculated. The results (table II) indicate that the T_c and the T_t increase as the stage of maturation progresses. No significant differences in slope were observed between BM cells from normal and porphyric calves.

Discussion

The influence of synchronization of cell cultures in studies of this type should be recognized [9]. In the present study, the data for the initial 6 h of incubation are very likely to have been influenced by the synchronization induced by the chilling period. This would mean that the observed changes in cell multiplication and differentiation take on significance mainly after this 6-hour synchronization period.

The initial Bn/Pn/LPn/On ratios were 1/3/8/19 in the normal as compared to a ratio of 1/3/12/32 in the porphyric. The most likely explanation for the greater number of more mature cells in the porphyric is a delay in the maturation of On to the reticulocyte which was induced by the porphyrins in a manner similar to the delay in maturation of the reticulocytes seen in porphyria [11].

The On increased at a rate of 47 cells/mm³/h in the normal and 50 cells/mm³/h in the porphyric. In order to replace the total cell population at this rate in each of the respective *in vitro* flasks, it was estimated that 42 h would

be required for the normal and 74 h for the porphyric cells. From a plot, the slope of the rising On curve was 1.91%/h in the normal and 1.14%/h in the porphyric. These figures give replacement times of 52 h for the normal and 87 h for the porphyric cow which compares favorably with the previous estimate. Therefore, the T_1 of the normal On is about 42-52, and about 74-87 h in the porphyric cow. This longer T_1 could again be due to a delay in maturation of the On.

The cell maturation and kinetic data involving the younger erythrocyte precursors, the Bn, Pn, and LPn, however, were not significantly different from normal (table II). The ratios for T_e for the Bn, Pn, LPn are 7.11, 21 for the normals and 8.11, 18 for the porphyrics. The only difference observed among these cell types was the earlier attainment of peak activity and a shorter plateau period in the porphyrics. Evidence of increased activity is to be expected in accelerated erythropoiesis [2] such as is consistently observed in porphyric cattle with or without frank anemia [3]. Under conditions of less marked erythropoiesis, however, the turnover of labeled cells may not be increased enough to show a change of T_e .

Comparison of the Bn, Pn, LPn ratios with reported ratios in other species [1, 5] indicates that the T_e and T_1 of cows is shorter for the younger cells and longer for the more mature cells. These variations among species are not surprising since the life span of the erythrocyte and the corresponding rates of erythropoiesis are different among the species. The percent-labeled cells provide an approximation of the DNA synthetic time (T_s) in relation to the cell cycle time (T_c) [8], i.e., $T_s/T_c = \% \text{ labeled cells}$. The T_s would be greater in relation to the T_c in the youngest cells since these have the highest % labeling and would be proportionately less in the older cells. Since the grain counts per labeled cell are indicative of the relative rates of DNA synthesis [8] the higher grain counts found in the younger cells would further support the conclusion of a greater proportionate DNA synthetic activity and T_s in the younger cells. Using the values for T_e and the average % labeled cells at 6 to 9 h, the T_s for the Bn, Pn and LPn are 6.8, 13 h in the normal and 8.9, 12 h in the porphyrics. These times are in close agreement with those obtained by others [6, 8, 12, 13]. Also, based on the T_1 , the total time for transit of erythroid cells in the normal BM would be between 100-110 h. This figure compares well with the 72-96 h required for the first appearance of ^{59}Fe -labeled erythrocytes in normal calves after injection of ^{59}Fe and the 8-10 days required to reach maximum incorporation [3].

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Handbuch der allgemeinen Pathologie, vol 7/3 Immunreaktionen Redigiert von
A. STUDER und H. COTTIER Springer, Berlin 1970 XIV + 557 pp 129 fig
DM 265 -/US\$ 72 90

Der vorliegende Band stellt mehr dar als «nur» einen Teil des Handbuches der allgemeinen Pathologie. Die darin enthaltenen Übersichtsarbeiten sind so aus gezeichnet geschrieben und so gut zusammengestellt, dass sie das Interesse nicht nur der Pathologen sondern aller Biologen im weitesten Sinne die sich mit Immunologie beschäftigen wecken sollten. Der Band gibt in erster Linie eine Übersicht über die physiologischen zellularen Vorgänge die die Immunisierung und die Immunität ausmachen wie sie sich in den letzten Jahren herauskristallisiert haben. Dabei werden auch Hypothesen formuliert und Fragen aufgeworfen. Alle Autoren sind aktiv forschend auf den verschiedenen Gebieten und wurden nach ihrer Befähigung und ihren Kenntnissen gewählt. Dies erklärt auch die Zweisprachigkeit des Buches (Englisch/Deutsch). Jeder drückt sich am besten in seiner Muttersprache aus. Das Lesen vermittelt nicht nur Wissen sondern regt an. Themen wie Makrophagen, Thymus usw. die heute im Vordergrund stehen werden in verschiedenen Kapiteln unter anderen Hauptthemen wiederholt hervorgehoben.

Die Gliederung des Bandes zeigt eine innere Logik. Im ersten Kapitel werden die Herkunft, Entwicklung und Funktion der Makrophagen untersucht. Der Zellen die vielleicht als erste mit Antigenen in Kontakt kommen und deren Bedeutung und Physiologie in vielem noch unbekannt sind. In den beiden nächsten Kapiteln werden das immunbiologische zelluläre System seine Ontogenese und seine Bestandteile im Hinblick auf die Immunantwort untersucht. Dabei werden auch in der zweiten Arbeit die Störungen der Entwicklung die zu den verschiedenen Defektimmunopathien führen untersucht. Das folgende Kapitel der zellulären Grundlagen der immunbiologischen Reizantwort behandelt zuerst in einem Beitrag die zellulären und molekularen Erkennungsmechanismen, vorgang der Immunantwort. Dann behandelt ein Beitrag die primäre humorale Immunreaktion und ihre Unterdrückung die Toleranz. Die sekundäre oder anamnestiche Reizantwort folgt im nächsten Beitrag. Anschliessend werden hauptsächlich Probleme der zellständigen Immunität behandelt. Diese eben erwähnten Arbeiten bilden den Hauptteil des Buches und schliessen den Weg des Antigens ein wobei alle den den Hauptteil des Buches und schliessen den Weg des Antigens ein wobei alle Etappen die auf den Zellen des Immunsystems im weitesten Sinne beruhen gehend und ausgiebig besprochen werden.

Die beiden letzten Kapitel des Bandes sind ein wenig ausserhalb des vorher gehenden Gebietes jedoch auch von grossem Interesse. Zuerst ein Kapitel über die Rolle der Mastzellen bei akuten Überempfindlichkeitsreaktionen. Ein Thema das oft vor lauter Lymphozyten zu kurz kommt. Der Band schliesst mit einem Kapitel über die Bedeutung der Immunologie in dem Gebiet der Onkologie wobei in der ster Linie die tumorspezifischen Antigene behandelt werden.

Der vorliegende Band gehört in die Handbibliothek jedes biologisch interessier ten Institutes.

T. VISCHER Basel

PHA Short-Term Culture of Lymphocytes in Acute Leukemia during Remission

Relation with Therapy

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Abstract The PHA induced blastogenesis in lymphocyte cultures of acute leukemia patients in remission was investigated. Lymphocytes of patients untreated for more than 2 weeks showed normal blast transformation. Lymphocytes of patients in treatment with 6-mercaptopurine or methotrexate showed in some cases normal blast transformation and in other cases lack of blastogenesis. It seems that in these patients the lymphocytes are sensible to the immunodepressive action of the mentioned antimetabolites, whereas in the other patients the lymphocytes are resistant to that action.

Key Words

Acute leukemia
Autoradiography
Cell culture
Immunosuppression
Leukemia treatment
PHA stimulation

Several authors have reported on the treatment [6] and the cell biology of acute leukemia during remission [5, 9, 13-15]. It seems well established that, in order to prolong remission, therapy must be continued with a cyclic use of agents, that can interfere with vital processes of normal cell like lymphocytes. An important expression of biological activity of such cells is the blast transformation in presence of phytohaemagglutinin (PHA) [12] that causes morphological and biochemical modifications [2, 4, 7, 8]. A 72 hour culture of lymphocytes from normal subjects, in presence of PHA, yields 60-70% of blast-like cells, 20-25% of prolymphocytes, and only about 10% of lymphocytes [1]. One of the most important biochemical modifications is the RNA synthesis in prolymphocytes and in blast-like cells, which becomes an almost continuous process [10].

In this paper we report on the PHA action on short-term cultures of lymphocytes of acute leukemia patients in remission, its relation with

therapy and the metabolic activity modification of nucleic acids in cultured cells studied by the tritiated uridine incorporation

Material and Methods

The observations concern 4 normal subjects and 28 acute leukemic patients in therapeutically obtained remission of which 4 were not treated for more than 2 weeks 14 were in treatment with 6MP (6-Mercaptopurine) and 10 in treatment with MTX (methotrexate)

Venous heparinized blood (10 ml) was collected in a cone tube and then left to sediment at 4°C for 1 h The blood was centrifuged at 500 rpm for 5 min The supernatant (10 000 white cells/mm³) was removed and transferred in silicon coated universal bottles containing TC 199 (4 ml/ml of plasma) and PHA (0.03 ml/ml solution)

Following a 72 hour incubation at 37°C, tritiated uridine (specific activity 1 Ci/mm) at 5 μ Ci/ml concentration was added to the culture for an additional hour of incubation From the ³H uridine labelled samples cells were sedimented by centrifugation and smears were made on gelatin coated slides All smears were fixed with methanol and coated with nuclear emulsion Ilford K5 After the required exposure time at 4°C, autoradiographs were developed and stained with Giemsa The leukocyte modification was estimated by counting the lymphocytes prolymphocytes and blasts A minimum of 200 cells for each sample was scored Nuclear and cytoplasmatic grain counts were performed in lymphocytes prolymphocytes and blast cells

Results

The results are reported in tables I-IV By comparing the results of table I and II it appears that lymphocytes of patients in remission un

Table I PHA short term culture of lymphocytes in control subjects

Case No	Sex	Prolymphocytes ¹		Blast like cells ¹	
		%	m g c	%	m g c
1	M	36	20	57	70
		15	20	61	80
2	F	16	20	65	100
3	F	20	20	64	60
4					

¹% = percentage of prolymphocytes and respectively blast like cells
m g c = mean grain count/cell

Table II Acute leukemic patients in first remission not treated for 2 weeks

Sex	Age, years	Survival months	Duration of remission ¹		Leukocytes ²			Infectious disease	Lymphocyte PHA-cult.			
			Before months	After, months	Number per mm ³	N	L		Prolymphocytes %	m g c	Blast like cells %	m
F	10	>24	12	>12	5 000	48	52	-	30	20	66	8
F	11	>84	84	> 6	5 000	58	42	-	23	30	62	10
F	3	13	5	7	4 200	60	40	chicken pox	22	20	63	8
F	4	>11	9	> 1	4 200	38	62	-	25	20	60	11

¹ It indicates the duration of remission before and after the aspiration of blood for culture

² N=neutrophils L=lymphocytes

³ %percentage of prolymphocytes and respectively blast like cells m g c mean grain count/cell

treated for more than 2 weeks, behave almost in the same way as control lymphocytes

From table III, it can be seen that 8 out of 14 patients treated with 6MP give the same results ■ controls, while in the other 6 the PHA-responsivity is sharply decreased. Autoradiographic investigation indicated that ³H uridine incorporation is high in blast like cells, lower in prolymphocytes and completely absent in lymphocytes.

Results concerning lymphocyte cultures of patients in remission, treated with MTX are reported in table IV. In 5 cases (No 1-5) the percentage of phyto-sensitive cells was normal, with high labelling incorporation, while in 5 other cases (No 6-10) a small percentage showed blast transformation. All tables also include clinical and hematological data useful for the interpretation of culture results.

Discussion

The reported experiments show that lymphocytes of acute leukemia patients in remission are capable of blast transforming in presence of PHA. Trinitated uridine incorporation shows activation of nucleic acid synthesis in all blast transformed cells. In patients treated with therapeutic agents like 6MP and MTX, however, we found a variable responsiveness.

Table III Acute leukemic patients

Case No	Sex	Age, years	Re-mission	Survival, months	Duration of remission ¹	
					Before	After
1	F	7	1st	>36	2 years	~ 1 year
2	M	7	1st	>18	10 months	> 8 months
3	F	2	1st	>13	6 months	10 months
4	F	4	1st	>12	5 months	> 6 months
5	F	3	1st	>12	3 months	~10 months
6	F	11	1st	> 7	1 month	3 months
7	F	5	1st	>11	6 months	> 5 months
8	F	3	1st	>13	1 year	~ 1 month
9	F	5	2nd	>11	1 month	10 months
10	F	4	1st	>11	10 days	10 days
11	M	2	1st	>13	6 months	~ 6 months
12	F	5	1st	> 8	3 months	> 5 months
13	M	6	2nd	30	12 months	6 months
14	M	10	1st	10	1 month	2 months

¹ It indicates the duration of remission before and after the aspiration of blood for culture

² N=neutrophils, L=lymphocytes, EO=eosinophils, M=monocytes

Table IV Acute leukemic patients

Case No	Sex	Age, years	Re-mission	Survival, months	Duration of remission ¹	
					Before	After
1	F	5	2nd	>11	3 months	~10 months
2	M	8	1st	~16	11 months	~ 6 months
3	F	10	1st	~13	5 months	> 8 months
4	F	5	2nd	~13	10 months	2 weeks
5	F	4	1st	~18	17 months	~ 1 month
6	F	2	1st	~13	9 months	> 5 months
7	F	3	1st	~12	9 months	> 4 months
8	M	3	2nd	~25	11 months	> 8 months
9	M	2	1st	~20	19 months	~ 1 month
10	M	3	2nd	>12	10 months	~ 2 months

¹ It indicates the duration of remission before and after the aspiration of blood for culture

² N=neutrophils, L=lymphocytes, EO=eosinophils, M=monocytes

in remission treated with 6MP

Leukocytes*					Infectious disease	Lymphocyte PHA-culture ^a			
Number/ N, mm ³	* _o	L, %	EO, M, % % _o			Prolympho- cytes		Blast like cells	
						%	m g c	%	m g c
3 900	54	43	3		viral hepatitis 6 months before	25	40	60	100
9 800	50	50			rubella 1 month after	15	20	81	100
4,200	33	56	1		—	20	60	70	80
4,200	32	63	2	3	—	33	30	53	50
2,800	53	45		2	—	33	40	60	100
2,000	20	80			viral hepatitis 1 month after	80	20	60	50
5 000	64	35		1	—	20	20	65	70
9 400	45	55			rubella, 5 months before	30	20	81	60
8 700	48	51		1	—	24	30	10	40
5 600	62	48			—	12	90	13	81
6 460	46	54				40	14	5	60
8 400	66	30		4		30	40	33	60
2,200	48	52				20	60	2	60
3 000	81	40				32	30	10	50

^a%, percentage of prolymphocytes and respectively blast like cells m g c = mean grain count cell

in remission treated with MTX

Leukocytes ^a					Infectious disease	Lymphocyte PHA-culture ^a			
Number/ mm ³	N %	L %	EO M, %			Prolympho- cytes		Blast like cells	
						%	m g c	%	m g c
10 200	68	30		2		21	30	57	81
3 400	81	52				17	23	77	100
6 800	64	36			-	30	60	55	100
8 800	20	78		2	chicken pox 1 week after	35	30	60	70
6 800	40	57		3	-	22	30	56	60
7 800	28	71		1	rubella 1 month before	46	40	16	50
6 400	49	47	2	2	chicken pox 1 week after	11	12	14	25
8 200	81	52				40	30	10	80
8 400	48	52				20	40	2	50
4 600	34	66				30	30	4	40

^a%, percentage of prolymphocytes and respectively blast like cells m g c = mean grain count cell

HERSH and OPPENHEIM [11] observed inhibition of lymphocyte transformation by PHA, during 6MP and MTX therapy in subjects with neoplastic diseases. As these authors point out, their results indicate immunodepressive action of the above mentioned antimetabolites, since blastogenesis *in vitro* is related to antibody formation [3].

From tables III and IV it can be seen that the mentioned 6MP and MTX immunodepressive action is present in some patients whose lymphocyte PHA cultures exhibit very low percentage of blast like cells. However, this action has not been seen in lymphocyte cultures of other patients treated in the same way. This different behavior cannot be explained in terms of a difference of clinical and hematological parameters. One may thus assume that lymphocytes resistant to the immunodepressive action of 6MP and MTX are present in some patients. The factor involved in the lymphocyte insensitivity to the immunosuppressive action of antimetabolites is still obscure. It is of interest to note that clinical data show that the therapeutic action of antimetabolites was always found to be efficient.

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Maturation of Leukaemic Blast Cells¹

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Abstract The phagocytic ability and the proliferative activity of leukaemic blast cells were investigated in 24 patients with acute leukemia. A certain phagocytic activity was found in most cases of acute leukaemia. The highest phagocytic activity occurred in monocytic leukaemia. Blast cell populations with a relative high phagocytic activity showed a low proliferative activity and only a few cells in DNA synthesis. The blast cells with phagocytosis are more mature cells. We got the impression that they are resting cells and possibly end cells.

Key Words
Cell maturation
Autoradiography
Cytophotometry
Leukemic cells
Phagocytosis

In vivo investigations with ³H-thymidine [1, 2, 3] indicate that leukaemic blast cells do not represent a uniform cell population. They confirm the existence of blast cell populations with different generation times in the same patient. KILLMANN [3], GAVOSTO *et al.* [4], BOLL [2] and others demonstrate models of leukaemic blast cell development with 2 leukaemic cell pools: one with a high labelling index and the other with a low labelling index. The latter may be resting cells or cells with a very long cycle time.

One attribute of mature cells and a sign of functional differentiation is the ability of phagocytosis [5-9]. Normal mature blood cells, for instance segmented neutrophils or monocytes, are able to phagocytize *in vivo* and *in vitro* bacteria or Indian ink particles. Until now no attempt has been undertaken to prove systematically the possibility of phagocytosis in blast cells [10] and to compare the findings with morphological signs of cell maturation and cell kinetics.

¹ Supported by Deutsche Forschungsgemeinschaft. Paper given at the 3rd Meeting of the European Study Group for Cell Proliferation, Varna 1970.

To answer the question of blast cell maturation we first examined, whether there exist some leukaemic blast cells with the ability of phagocytosis. Then we carried out autoradiographic investigations after ^3H thymidine incubation and DNA measurements in blast cells of the same patients.

Materials and Methods

Investigations of 28 cases of acute leukaemia in different stages of disease. The type of leukaemia was determined with the aid of cytochemical methods [11-12].

Phagocytosis Defibrination of 5 ml vein blood by shaking with glass beads. Sixty minutes incubation of 4 ml defibrinated blood with 4 drops of Indian ink Pelkan Nr 541 in 37°C preparation of smears from the upper part of the tube which contains a leukocyte concentrate.

Autoradiography After ^3H thymidine incubation [13-14] calculation of labelling index in 1 000 blast cells in 2 corresponding smears.

Cytophotometry Semiquantitative DNA estimation after Feulgen staining [15-16]. Photometry of 50-350 blast cells per smear. All measured blast cells were divided with the aid of graphical methods into groups of cells in G_0 or G_1 phase with a $2c$ DNA content and of cells in DNA synthesis with $2c-4c$ DNA content.

Results

Normal segmented neutrophils or monocytes show a phagocytosis of many large Indian ink particles all over the cytoplasm (fig 1a). After incubation of peripheral blood of leukaemic patients we were able to de-

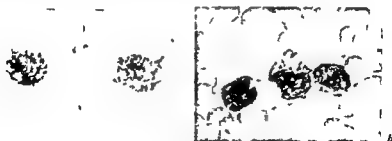


Fig 1 Phagocytosis of Indian ink particles in normal monocytes and segments (a) and in a leukaemic blast cell (b). Many large grains all over the cytoplasm of normal blood cells, only a few Indian ink grains in the whole cytoplasm of the leukaemic blast cell.

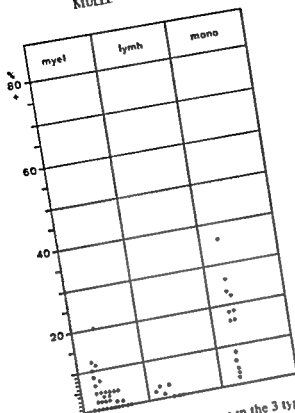


Fig 2 Number of blast cells with phagocytosis in the 3 types of leukaemia

tect some blast cells which show a phagocytosis of Indian ink particles (fig 1b) As a rule these blast cells with phagocytosis possess a stretched nucleus and only small nucleoli In myeloblastic leukaemia they have a distinct cytoplasmic granulation and their cytoplasm is not coloured so deeply blue after panoptic staining All leukaemic blast cells with phagocytic activity have only a few Indian ink grains in the cytoplasm This criterion may be helpful – besides other morphological criteria – for the distinction of blast cells and monocytes because monocytes are full of Indian ink grains

The amount of blast cells with the ability of phagocytosis is low as can be seen in figure 2 Their percentage is a little higher in the monoblastic leukaemias than in the other forms This may be due to the fact that our cases of monocytic leukaemias had relatively mature blast cell populations

The proliferative activity was calculated in 24 out of the 28 cases of acute leukaemia, partially several times in the course of the disease [20]

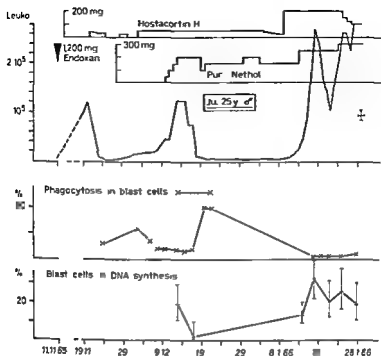


Fig 3 Leukocyte curve (above) phagocytic activity (middle) and blast cells in DNA synthesis (below) in a case of acute leukaemia (Ju 25 y)

The percentage of blast cells in DNA synthesis in a leukaemic cell population seemed to be inversely proportional to the phagocytic ability. We found a blast cell phagocytosis of Indian ink particles in up to 2% in 20 investigations and in more than 2% in 16 investigations. Among these, the first group had 6 and more percents of blast cells in DNA synthesis more frequently than the second group ($p = 0.1$). We found an especially high fluctuation of the percentage of cells in DNA synthesis during relapse and remission in 2 of our leukaemic patients. One of these cases may elucidate the connection between the amount of DNA synthesis and the ability for phagocytosis in blast cells (fig 3) demonstrating the findings of a 25 year-old man with an undifferentiated acute leukaemia. The phagocytic activity is in an inverse correlation with the extent of DNA synthesis of blast cells in this case. A high synthetic activity in relapse is connected with a low phagocytic activity.

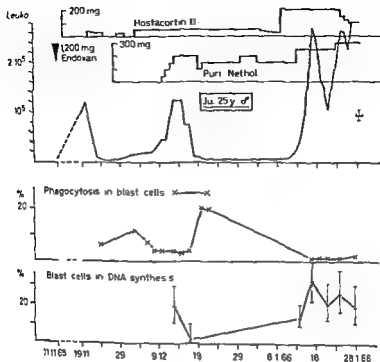


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Conclusions

The model of leukaemic cell proliferation of some authors includes 2 blast cell pools from which the first may be a pool of cell proliferation. This first pool shows a high amount of blast cells in DNA synthesis as a sign of a high proliferative activity. The other blast cell pool is a pool of cell maturation. Cell morphology confirms that at least a part of the blast cells show a tendency toward maturation [17]. Some blast cells have smaller nuclei, small or not visible nucleoli and clear cytoplasm in panoptic stainings, attributes of a certain stage of maturation in normal haemopoiesis.

It is in discussion whether all the nonproliferating blast cells are resting cells with a latent potential for division or rather end cells which have lost for ever the capacity to proliferate and are, therefore, destined to die.

We found that some leukaemic blast cells are able to phagocytose Indian ink particles to a certain extent. Similar findings were made by STRUMIA [1937] [6] and by RIND [1958] [18]. Blast cells with phagocytic activity very often have morphologic signs of more mature cells. After comparison of the proliferative activity, calculated by means of ^3H -thymidine autoradiography or DNA estimations and the ability of phagocytosis we found more frequently a distinct phagocytosis in blast cells in cases with a low proliferative activity of leukaemic blast cells than in cases with a high proliferative activity. The highest percentage of blast cells in DNA synthesis was 34%. No single blast cell out of this population shows a phagocytosis of Indian ink particles. Blast cells with a phagocytosis of Indian ink particles which may be a sign of a certain functional maturation are, therefore, connected with a morphological feature of maturation and frequently with a diminished proliferative activity of the population. These findings confirmed the concept that resting leukaemic cells with a diminution of DNA synthesis are at least more mature cells [19] and we got the impression that resting cells will become end cells.

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Granulocyte Function in Patients with Brain Damage and Anoxia

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Abstract Granulocytes obtained from patients with anoxia and with brain damage showed a consistent impairment of phagocytosis and adhesiveness. This phenomenon was not reversed by the addition of fresh human serum. These observations reveal a relationship between *in vivo* anoxia and brain damage with granulocyte function.

Key Words

Anoxia
Brain damage
Granulocyte adhesiveness
Granulocyte phagocytosis
Leukocyte isolation

During emergency tissue-typing for cadaver renal allotransplantation the relative inefficiency of methods for preparing pure lymphocyte suspensions from venous blood became apparent [1]. One difficulty was the persistence of granulocytes in the final suspensions resulting from the failure of these cells to adhere consistently to nylon wool fibres used in the standard technique. We were interested to find that it was granulocytes obtained from patients with brain damage and anoxia which were poorly adhesive while similar cells from patients with a variety of advanced non neurological conditions behaved normally. A satisfactory solution to this problem was found by applying Boxum's [2] flotation method for lymphocyte separation to tissue typing [1, 3, 4, 5].

Experiments reported here demonstrated that diminished granulocyte adhesiveness was closely associated with reduced *in vitro* phagocytic activity. Granulocyte adhesiveness in patients with recent brain damage was not increased by incubation with complement in the form of fresh normal ABO compatible serum. An acquired *in vivo* cellular defect is postulated to account for the impairment of the ability of granulocytes from brain damaged patients to adhere and act as phagocytes *in vitro*. Our evidence suggests that these abnormalities may result from tissue anoxia. However, it is difficult to

exclude secondary brain damage resulting from anoxia as the causative factor

Materials and Methods

Lymphocyte suspensions were obtained from defibrinated blood samples from a total of 11 normal volunteers, 7 patients with brain damage, 10 patients with severe central anoxia and 8 patients with a variety of acute surgical and medical problems but who had no brain damage and were not anoxic. The method used for preparing lymphocyte suspensions involved dextran sedimentation and incubation with nylon wool [6]. Granulocyte adhesiveness was assessed by counting the number of granulocytes remaining in the final lymphocyte suspensions.

Granulocyte adhesiveness estimated in this way was compared with phagocytic activity in 6 normal controls and 3 patients. A modification of the method used by BRAMPT [7] was used to assess phagocytic activity. 2 ml defibrinated blood were incubated with 1 ml yeast suspension (40 000 particles/ml) at 37°C for ½ h. The dextran sedimented white cell suspension was cytocentrifuged at 400 rpm for 15 min and the number of yeast particles contained in 100 granulocytes was counted. The result was expressed as the mean number of particles/granulocyte – the phagocyte index.

The effects of incubating with fresh human complement were investigated using granulocytes obtained within 24 h of operation from patients with cerebral tumours. Leukocytes and serum were also obtained from 3 normal ABO compatible control individuals. Normal cells with patients' serum and patients' cells with normal serum were incubated at 37°C for 30 min before incubation in the usual way with nylon wool. In each case the serum was obtained within 2 h of use.

Results

Figure 1 shows the percentage granulocyte contamination of lymphocyte suspensions prepared from defibrinated blood by dextran sedimentation and incubation with nylon wool. Blood samples obtained within 24 h of brain damage yielded lymphocyte suspensions with 14–43% granulocyte contamination and 12–75% contamination when blood was obtained from anoxic patients. This contrasts sharply with the normal values and with the low granulocyte contamination (0–7.5%) obtained from control patients with miscellaneous conditions not associated with brain damage or anoxia (table I).

Table II is a comparison of adhesiveness and phagocytic activity in 6 normal controls and 3 patients with anoxia. There is a marked correlation between adhesiveness and phagocytic index.

Table III shows the effect of incubating with complement, granulocytes obtained within 24 h of operation from patients with cerebral tumours and

Table II Comparison of granulocyte adhesiveness and phagocytic index in anoxic patients

No	Granulocyte contamination of lymphocyte suspension %	Phagocytic index	pO ₂ mm Hg
Normal controls			
1	1	1.5	normal
2	3	2.3	normal
3	6	1.7	normal
4	3	3.2	normal
5	5	2.9	normal
6	7	1.9	normal
Anoxic patients			
1 ¹	25	0.8	55
2 ¹	20	0.8	60
3 ²	70	0.2	75

¹ Brain damage
² Acute LVF

Table IIIa Effect on granulocyte adhesiveness of incubating leukocytes from brain damaged patients with autologous serum and serum from normal ABO compatible volunteers

Patient	Granulocyte adhesiveness %	
	Autologous serum	Serum from normal ABO compatible volunteer
A	50	52
B	65	25
C	12	29

Patients A = Glioma of anterior fossa B = Glioma of posterior fossa C = Frontal meningioma.

the results of control experiments with 3 normal ABO compatible volunteers. None of the serum samples from the patients in the test conditions used (incubation at 37°C for 30 min) reduced the adhesiveness of granulocytes from the normal controls and, except possibly for patient B, no improvement in adhesiveness occurred when granulocytes from the patients were

Table 111b Effect on granulocyte adhesiveness of incubating leukocytes from normal volunteers with autologous serum and serum from ABO compatible brain damaged patients

Normal volunteer	Granulocyte adhesiveness %	
	Autologous serum	Serum from ABO compatible brain damaged patients
1	10	12
2	12.5	4
3	11	5

incubated with fresh control serum. This observation confirms our tissue typing experience working with material from brain damaged patients when on many occasions the incubation of granulocyte contaminated lymphocyte suspensions with fresh human serum and nylon wool did not result in the removal of the granulocytes.

Discussion

It is generally believed that adequate levels of complement (and opsonins) are necessary for granulocyte phagocytosis to occur [9] but our experiments in which ABO compatible serum was incubated with leukocytes from brain damaged patients do not suggest that the failure of adhesiveness of granulocytes is due to deficiency of circulating complement. It is possible that damaged brain releases an inhibitor substance capable of impairing adhesiveness and phagocytosis. The impairment of adhesiveness and phagocytosis which we have detected in granulocytes from patients with anoxia suggests that reduced pO_2 may play a primary role and it is perhaps significant that many of our brain tumour patients were anoxic because of depressed respiration or postoperative problems. Indeed it proved extremely difficult to find patients with brain damage who had not had some degree of anoxia.

It has been shown [8] in a study of metabolic changes occurring during the ingestion of particles by granulocytes that phagocytosis occurs equally well under anaerobic and aerobic conditions. However, our experiments suggest that *in vivo* exposure to anoxia may affect the subsequent adhesive

and phagocytic properties of granulocytes. On the other hand anoxia may play a more indirect role by producing brain damage.

Whether brain damage or anoxia or both are responsible it is evident that the phagocytic and adhesiveness properties of granulocytes are impaired in these patients. This phenomenon came to our attention as a result of problems encountered during the emergency tissue typing of cadaver donors using lymphocyte suspensions but it is evident that the role of granulocytes in the resistance to infection by patients with anoxia and brain damage merits further study.

Acknowledgements We are grateful to Prof M. C. G. ISRAELS and other clinicians in Manchester Royal Infirmary for making available blood specimens. We are also grateful to Mrs R. HARRIS for her secretarial assistance. We acknowledge the financial help given to Dr R. HARRIS by the Board of Governors Research Grants Committee, United Manchester Hospitals and the help with illustrations given by Dr R. OLLERENSHAW.

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Diagnosis of Quinine Hypersensitivity, Use of Platelet Factor 3 and Acid Phosphatase Availability Tests

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Key Words

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Recently, we had the opportunity to investigate 2 patients having experienced in the past an allergic thrombocytopenia induced by quinine. In order to demonstrate the hypersensitivity due to quinine we studied the platelets of these patients under various experimental conditions including the platelet factor 3 availability test, the inhibition of clot retraction and the demasking of the acid phosphatase activity of the platelets. We have also investigated the action of Ca on the last reaction.

Case Reports

Case 1 Patient E. P., a 39 year-old male suffered in 1954 from severe bleeding, characterized by melaena, haematemesis and large suffusions due to an almost complete loss of platelets a few hours after ingestion of quinine-containing tablets. He recovered quite rapidly in about 1 week. In 1966, 1 h after drinking of quinine-containing tonic water he had shivers, vomiting, haematuria, gingival bleeding and collapse. The platelet count was $80\,000\text{ mm}^3$. He recovered after several days of prednisone therapy.

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During the present study both patients were examined 3 times over a period of 6 months and were found in good health with normal haematological findings.

Material

Citrate solution 3.8% trisodium citrate $5\frac{1}{2}\%$ H_2O EDTA solution 1 percent solution in 0.7% NaCl. Tris buffered saline 0.515 M Tris (hydroxymethyl) amino-methane in physiological saline. The pH is adjusted to 7.4 with 3 N HCl. Heparin, Liquemin Roche 5000 U USP/ml. Calcium chloride 0.1 M and 0.025 M CaCl_2 solution. Stypven, Russell's viper venom (Burroughs Wellcome). *p*-Nitrophenyl phosphate (Merck) a 150 mg% solution in 0.05 M citrate buffer at pH 5.5 has been used as substrate in the assay of acid phosphatase. Drugs: 2.5 or $7.0 \cdot 10^{-4}$ M solution in Tris buffered saline were prepared with the following drugs: quinine dihydrochloride, codeine phosphate, caffeine, paracetamol and ascorbic acid. These solutions were diluted 10 times with PRP before testing (1 part drug solution + 9 parts PRP). Platelet rich plasma (PRP): citrate or EDTA plasma were prepared by adding 9 parts of whole blood to 1 part of the anticoagulant in siliconized tubes. The blood was then centrifuged for 10 min at 200 g and 4°C . The platelet count in PRP is performed by means of the Unopette System (Becton, Dickinson and Company) and phase contrast microscopy. It varied between 360 000 and 420 000/ mm^3 .

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Platelet Factor 3 (PF 3) Assays

(a) Stypven method was performed according to BURGESS *et al* [2].

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plasma and the mixture recalcified. The restprothrombin is then determined after stopping the reaction with citrate and removal of the clot according to the original method [15].

Determination of the Acid Phosphatase (APh)

The acid phosphatase activity was assayed as previously described [16]. In some experiments the assay was made in presence of CaCl_2 to study the effect of Ca ions on the demasking of the acid phosphatase. The assays were performed as follows: 0.15 ml 0.1 M CaCl_2 containing 15 units heparin were added to 1 ml citrated normal or patient's PRP followed by 0.1 ml of the tested drug or Tris saline. In control experiments the calcium chloride solution was replaced by 0.15 ml Tris saline containing 15 units heparin. Aliquots (0.2 ml) of these mixtures were incubated at 37°C in a water bath. At various time intervals APh activity was determined as usual.

Determination of the Clot Retraction

0.5 ml PRP containing the investigated drug or Tris buffered saline are recalcified with 0.1 ml 0.1 M CaCl_2 . The clot retraction is measured after 60 min incubation at 37°C . At 100% retraction the length of the clot is reduced by half.

Results

Demasking of Platelet Factor 3 Activity

The effect of quinine on the Stypven time after incubation of PRP with the drug is shown in figure 1a and b for both patients. In both cases one

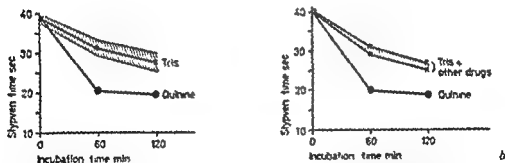


Fig 1 Effect of quinine on the Stypven time in quinine-sensitized patients. The hatched area represents the range obtained with PRP of healthy persons with either quinine or Tris buffered saline. *a* (Case 1) \times = patient's PRP + Tris buffered saline, \bullet = patient's PRP + quinine (final concentration 2.5×10^{-5} M). *b* (Case 2) \times = patient's PRP + Tris buffered saline or drugs (see text), \bullet = patient's PRP + quinine (final concentration 2.5×10^{-5} M).

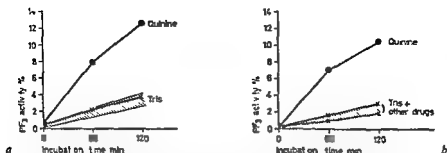


Fig 2 Effect of quinine on the demasking of PF 3 activity. Hatched area = normal range \times = patient's PRP with Tris buffered saline (a, case 1) or Tris buffered saline and other drugs (b, case 2) \bullet = patient's PRP + quinine (final concentration $2.5 \times 10^{-5} M$)

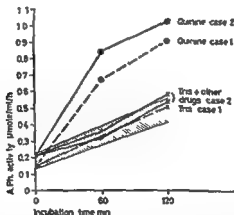


Fig 3 Effect of quinine on the demasking of acid phosphatase activity. Hatched area = normal range \times = PRP + Tris buffered saline, case 1, \times = PRP + Tris buffered or other drugs, case 2, \bullet = PRP + quinine, case 1, \bullet = PRP + quinine (final concentration $2.5 \times 10^{-5} M$) case 2.

observes a shortening of the stypven time at a final quinine concentration of $2.5 \times 10^{-5} M$. Lower quinine concentrations are practically without effect. In case 2, quinine only is effective while the other components of Sandofebral® are inactive (fig 1b). Using the quantitative assay for PF 3 [15] it can be demonstrated that 3-5 times more PF 3 activity is de-

Clot Retraction

When the PRP is recalcified immediately after addition of quinine there was no alteration of the clot retraction in the patient's PRP as compared with the control performed in presence of tris buffered saline. We found a slight decrease of the retraction in one assay only and after 120 min incubation of PRP in case 1 with quinine before recalcification.

Discussion

We have investigated 2 patients several months or years after they had experienced a quinine induced allergic thrombocytopenia. Even after these long intervals we were able to distinguish clearly between the PRP of the quinine-sensitive patients and normal PRP by means of *in vitro* experiments. In presence of quinine the PF 3 and acid phosphatase activities are made available in the patient's PRP. Similar results using the complement fixation test have been published by VAN DER WEERDT [21]. It may, therefore, be possible to detect drug dependent antibodies years after recovery from acute allergic thrombocytopenia. It seems that especially the acid phosphatase determination may be a very valuable tool for the detection of platelet changes under conditions where other methods fail. MÜLLER-ECKHARDT and LÜSCHER [13] point out the importance of Ca^{++} ions for the appearance of morphologic changes and for the retraction of platelet aggregates produced by various inducers. Our experiments made with the PRP of 2 quinine-sensitive patients seem to confirm this concept. Using the strong chelating agent EDTA we couldn't see any demasking of PF 3 and acid phosphatase activity in patient PRP, with citrate, a weak chelating agent, we observed a clear increase of PF 3 and acid phosphatase, whereas the demasked activity of the acid phosphatase was doubled upon addition of Ca^{++} as compared with citrated PRP. Together with Ca^{++} the patient's platelets developed a high activity of acid phosphatase. These results suggest that the citrate does not prevent the interaction between platelets and immune complexes.

It was naturally not possible to follow the evolution of PF 3 activity in presence of calcium because of the impossibility to block the coagulation without interfering with the PF 3 assay.

The action of quinine is quite specific and only much higher concentrations, $2.5 \times 10^{-3} M$, equivalent to 1 mg/ml of citrated PRP lead to a spontaneous demasking of acid phosphatase and PF 3 activity from normal PRP. Similarly HOROWITZ *et al* [7] found a PF 3 release at high quinidine concentrations only.

In attempt to explain our results it should be pointed out that the immune complexes are known to cause the platelet release reaction with subsequent degranulation and viscous metamorphosis [1, 13]. Although the pathophysiological mechanism of these alterations is not well understood, it appears that immune complexes can be phagocytosed by platelets [11, 12, 14]. The activation of platelet lysosomes as suggested by several authors [8, 11] seems to be a further step. In spite of some controversy about the nature of platelet lysosomes and the localization of platelet acid phosphatase activity [10, 18] recent studies — at least — do not exclude the presence of this activity in platelet lysosomes [3]. It is possible that the determination of acid phosphatase activity could be used as an indicator of the alteration of platelet lysosomes [8, 16]. The role of complement in the interaction of immune complexes with platelets is not yet clear. Some authors found its role negligible or questionable [11, 13, 22], some found it important [5, 19]. The enhancing action of calcium ions in the development of the acid phosphatase activity confirms the assumption that the complement may play a role in the platelet damage. These ions would allow the binding with the immune complexes. However, the failure of heparin, a known anticomplement agent added together with calcium in our experiment, to inhibit the demasking of acid phosphatase activity might speak against an interaction with the complement [20].

The enhanced availability of platelet acid phosphatase activity by immune complexes in presence of calcium may be due (1) to their phagocytosis by platelets causing a more pronounced lysosome activation, or (2) to the binding of complement on immune complexes with an increase of their toxic action on platelets. Experiments with the plasma of patients with a higher antibody titer could probably help to solve this problem.

It should be pointed out that the acid phosphatase activity measured in our experiments, also after addition of Ca is actually not released since it is still bound to platelet rests and aggregates and easily sedimentable with them. The term availability [9] or demasking [4] of acid phosphatase or PF 3 activity should be preferred to release to describe these changes. The changes produced by the immune complexes are quite similar to those due to the action of ADP, epinephrine, collagen and thrombin [9].

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Sickle Cell Trait in a Caucasian Population Proportion of Hemoglobin S in Hemolysates from Saudi Arabs

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Abstract The percentage of Hb S in hemolysates from 109 Saudi Arab males was found to range from 18-47%, with a mean of 33%, an obvious mode at 32-34% and clear skewing of the overall pattern toward lower values. A tentative explanation of this finding is the concurrence of the α thalassemia trait in this population which tends to reduce the percentage of Hb S in erythrocytes of double heterozygotes.

Key Words

α thalassemia
Caucasian population
Hemoglobin S
Saudi Arabs

Previous studies in the American Negro have suggested that the frequency distribution of the percentage of Hb S in the hemolysates from subjects with the sickle cell trait, tends to assume a bimodal pattern, with clustering at 34-36% and at 40-42% [3, 7, 12]. This contention has been challenged by a more recent study which showed an essentially unimodal distribution of Hb S percentage in hemolysates from Georgia Negroes, with clustering at 37-38% [13]. Investigation of 60 Brazilian Negro and mulatto subjects with the sickle cell trait disclosed a trimodal distribution of Hb S percentage, with clustering at 32-34%, 42-44% and 48-50% [8].

The purpose of the present communication is to report data on the proportions of Hb S and A in the blood of Saudi Arabs residing in the Persian Gulf area. This Caucasian population is known for the high frequency of the sickle cell trait [2, 4].

Subjects and Methods

Saudi subjects with the sickle cell trait were initially identified with the metabisulphite screening test [1]. The percentage of Hb S in hemolysates from these sub-

jects was determined by spectrophotometry of hemoglobin eluates on cellulose acetate strips (Oxoid or Sephadex III) following the separation of hemoglobin components by electrophoresis in a Gelman horizontal cell with 0.1 M Tris EDTA borate buffer at pH 8.6. Only unrelated, non anemic, adult male subjects were selected for this study. Most of the subjects were contacted in connection with a family study evolving from previous experience with family member(s) with sickle cell disease, or for certain clinical indications which included unexplained anemia, jaundice and/or bone pain, hematuria and jaundice.

Results

Hundred and nine Saudi males were included in this investigation, which was conducted from June 1966 through September 1968. The frequency distribution of Hb S percentage in hemolysates from this group showed considerable spread, from 18–47%. There was a suggestion of clustering at 25, 29 and 37–39%, with the most evident mode at 32–34%, and a mean of 33% for the group (fig 1). It is noteworthy that the distribution pattern was clearly skewed toward lower values, and the frequency in the 40–50% range was very low.

Comment

There are a number of possible explanations for the data exhibited from this study. The lack of a clear modal distribution in the frequency

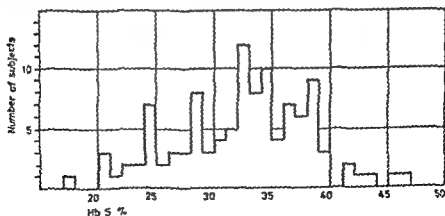


Fig 1 Proportion of Hb S in hemolysates from 109 Saudi males

of Hb S percentages may actually reflect a random selection of subjects, whereas previous publications have shown inclusion of a number of related subjects, and there is evidence that the Hb S percentage frequency distribution may be in a narrow range among subjects from the same family [3, 7]. Thus inclusion of several large families in this type of study would tend to produce a spurious aggregation of percentages, and possibly a multi modal pattern. Inclusion of anemic subjects may also distort the frequency distribution pattern [5]. Considering the early suggestion of ITANO [3] that the inheritance of the sickle cell trait involved the effect of multiple alleles, it follows that there could be substantial ethnic variation in the expression of the sickle cell trait, depending on allelic control of the rate of Hb S synthesis. It appears that the presence of the gene for α -thalassemia may suppress the level of β -chain variants in double heterozygotes, which in the case of the sickle cell trait would tend to reduce the percentage of Hb S [9, 10]. The α -thalassemia trait(s) is known to occur at a significant frequency within the Saudi oasis population along the Persian Gulf [6], and interaction with the sickle cell trait might be expected to occur frequently [11]. For the present, this is the most plausible explanation for the skewed distribution toward low values for Hb S percentages in the Saudi group described in this report.

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Study of Hemoglobins in a Population of Yucatan

Comparison with other Mexican Groups

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Abstract The results of a first investigation on hemoglobins of Maya Indians of the Yucatan Peninsula in Mexico are set out. This investigation confirms the very infrequent occurrence of hemoglobinopathy among the Indians of the American continent

Key Words
Hemoglobin types
Hemoglobinopathies
Mexico

With the help of the WHO, the Centre d'Hématologie of the French National Investigation Center carried out a study of a Maya population of Yucatan. The results of that study have already been expressed in global terms [2]. In this article we continue the analytical and comparative study of the hemoglobinopathies found by us and other investigators both in other regions of Mexico and in other regions of South America.

Material

Our investigation took place in the region of Peto, an important village in the center of Yucatan in the department of Merida (fig. 1). In the course of that mission we have studied the hemoglobin of 515 Mexicans: 413 Maya and 102 half caste (White/Maya crossbred).

Of those cases studied, 111 lived in towns of more than 10 000 inhabitants: Peto, Ticul, Campeche and Mérida. The others came from smaller villages, 376 from villages in the north of the country: Tixhualatun, Tixmehuc, Kimbila and Xchobil; 55 from villages in the south: Yaxcopil and Tzucacab. All age groups were represented in this sample.

Table I

Ethnic group	Number	AS	A ₂ >3.5%	F >2.5%	A ₂ +F	Total thalassemia
<i>Mayas</i>						
Towns with >10 000 inhabitants	36	-	1	1	-	2 0.0555
Northern villages	323	-	8 0.0247	2 0.0061	8 0.0247	10 0.0557
Southern villages	54	1	-	-	-	-
Total	413	1	9 0.0217	3 0.0072	8 0.0193	20 0.0484
<i>Half-caste</i>						
Towns with >10 000 inhabitants	48	-	1	3 0.0625	-	4 0.0833
Northern villages	53	-	-	-	1	1 0.0188
Southern villages	1	-	-	-	-	-
Total	102	0	1	3 0.0294	1	5 0.0490

Conclusions

The 2 groups studied are not exactly comparable, as the Maya population is 4 times greater than the population of half-caste. However, the overall frequencies of thalassemia are about the same in the 2 groups, 0.0484 and 0.0490. It must, however, be noted that in the 25 cases where the existence of β thalassemia was suspected, a conclusion is not possible in the absence of clinical, biological and family investigations. The Hb \bar{S} gene is not usually present in the Amerindians. The presence of an AS heterozygous individual can only be explained by a negroid contribution.

Among the other studies of other authors on hemoglobins in Mexican Indians, we must make mention of the works of LISKER *et al* [11-13],

those of MATSON *et al* [14, 15] and of SUTTON *et al* [24] on the Indians of Central America and of South Mexico. Those authors have not found hemoglobin anomalies, save a few cases of heterozygous AS due to a negroïd crossbreeding. However, in the course of an investigation carried out on 3,000 Indians, they have found in 5 subjects, a fast fraction near the Hb N which they named Hb Mexico, and in 1 subject, a slower hemoglobin than Hb A at alkaline pH and faster at acid pH. This last one they have named Hb Chiapas, after the name of the group where it was found. We have not been able to find any of those anomalies in the course of our investigation. It must be admitted, though, that our investigation was based on a more limited number of subjects. Generally speaking, it looks as if abnormal hemoglobins are a rare occurrence among American Indians not crossbred.

In Northern America SCOTT *et al* studied the Eskimos of Alaska [22], POLLIZER *et al* [17] 2 Indian population groups in North Carolina, the Cherokee and the Lumbee. There was no case of hemoglobinopathy among the Eskimos and rare cases of hemoglobin S or C in North Carolina, which were probably due to crossbreeding. Perhaps a few cases of hemoglobinosis D may be proper to pure-blood Indians, but such cases are always rare.

The populations of South America have been more extensively studied. ARENDS has studied the Indians of Columbia [1], ARENDS [2] and GUEGARA and ARENDS [9] the hemoglobins of Venezuelan Indian children. NEEL *et al* [16], TASHIAN *et al* [25] and TONDO and SALZANO [26] those of the Xavante and Caingang Indians of Brasil.

Our own team has carried out over the last 6 years a hemotypological study of the Indians of the High Plateau of Bolivia and of Peru [3, 8, 18, 19, 21] and another expedition was undertaken in 1962 by some of our group to study the Indians of French Guyana [4-7, 10]. In all those countries only those regions where crossbreeding with the negro population has been important show a certain percentage of abnormal hemoglobins, S or C (coastal regions of French Guyana for example or in Central America where paludism may have played a selective role regarding hemoglobin S, certain regions of Venezuela).

It is known that, apart from Hb E which without doubt appeared recently, mutations affecting the structure of globin chains are rare among the populations of the Far East which is the place of origin of the American-Indians inhabiting the New World. It is not accordingly surprising that the latter, when they are not a result of crossbreeding, show

normal α - and β -chains. It must be added that paludism, endemic to a low degree in South America through the absence of proper vectors, has not been able to play its selective role regarding the particular mutations which may have appeared in certain groups (Hb Mexico, Hb Chiapas).

Only thalassemia (β -thalassemia with Hb A₂) is observed rather frequently in Guyana [6], Brasil, Venezuela [9], Bolivia or in Peru [19]. But even in that case further studies are necessary to ensure an exact assessment of the distribution of that anomaly.

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I M. YOFFEY and F. C. COURTICE: *Lymphatics, Lymph and the Lymphomyeloid Complex.*

Das umfangreiche Werk behandelt in gemeinsamer Betrachtung das lymphatische System als ein ausgedehntes Netzwerk von Lymphgefässen mit Lymphflüssigkeit und Zellen, das verstreute lymphatische Gewebe und den lymphozytären Anteil des Knochenmarks, zusammengefasst unter dem Oberbegriff des lymphomyeloiden Komplexes. Im ersten Teil des Buches werden Physiologie und Pathologie der Lymphe abgehandelt. Der zweite Teil befasst sich mit den Lymphozyten und dem lymphatischen Gewebe, beginnend mit der Morphologie und Funktion der Lymphozyten und sich weiterhin erstreckend auf geneische Beziehung zwischen Lymphozyten und anderen Zellen, z. B. Plasmazellen und Makrophagen, die Antikörperbildung, die Entstehung von Lymphozyten, die Übergangszellen als mögliches Bindeglied zwischen Lymphozyten und Stammzellen undliesslich die Zellströme, die das lymphatische System bzw. das lymphomyeloide System integrieren.

Der Wert des Buches liegt in der koordinierenden Betrachtung des gesamten Gebietes durch zwei erfahrene Forscher auf den Gebieten des Lymphsystems [COURTICE] und der Lymphozyten [YOFFEY], so dass dem Leser die Zusammenhänge zwischen lymphatischem Gewebe, Lymphsystem und Knochenmark deutlich werden. Der Nachteil des Werkes, nämlich die Unmöglichkeit einer detaillierten Darstellung von Einzelfragen, tritt gegenüber dem Vorteil dieser übergeordneten Betrachtungsweise deutlich zurück. Die zunehmende Spezialisierung engt naturgemäss den Gesichtskreis eines Forschers ein, so dass gerade jetzt, wo das Lymphozytenproblem so sehr in den Vordergrund gerückt ist, diese Bearbeitung besonders zu begrüßen ist. Der Hamatologe hat Gelegenheit, sich mit den während einer langen Forschertätigkeit entwickelten originellen Auffassungen von YOFFEY auseinanderzusetzen.

H. BRÜCHER, Berlin

G. SCHÜTTLER: *«Platelets and the Vessel Wall-Film Deposition»* G. Thieme, Stuttgart 1970 183 pp

Dieses preiswerte Buch faßt die am Symposium der Europäischen Atherosklerosis Gruppe im Juni 1969 gehaltenen Referate von 25 namhaften, vorwiegend europäischen Forschern zusammen. Eine erste Gruppe von Arbeiten befaßt sich mit Fragen der Morphologie und Biochemie der Plättchen. An eine knappe, kritische Übersicht über die Physiologie der Thrombozyten (O'BRIEN) schliessen sich Arbeiten über die Beschaffenheit der Thrombozytenoberfläche und der subzellulären Strukturen, sowie über verschiedene Probleme des Stoffwechsels an. Das Referat von LÜSCHER über die verschiedenen Arten der Thrombozytenaktivierung leitet etwa ein halbes Dutzend Arbeiten über verschiedene Einflüsse auf die Thrombozytenfunktion (vor allem Aggregation und Adhäsion) ein. Der Hauptteil des Buches ist sodann den Beziehungen zwischen Thrombozyten, Gerinnungsbildung und Gefässwand gewidmet. Der erste Teil enthält eine grosse Zahl von Angaben, die sich zum Teil auf epidemiologische bzw. klinische Beiträge und im allgemeinen sehr knapp gezeichnete Zahl von Literaturangaben

Neue Erkenntnisse über die Thrombozytenfunktion und -beschaffenheit haben das Interesse an den Blutplättchen und ihrer Bedeutung für die Entstehung der Arteriosklerose neu geweckt. Zum Teil werden grosse Erwartungen in Bezug auf Behandlungsmöglichkeiten an diese neue Forschungsrichtung geknüpft. Gerade für Hämatologen ist es von besonderem Reiz, die Beziehungen zwischen Gefässendothelien und Thrombozyten näher kennenzulernen. Das kleine, sorgfältig redigierte Buch gibt einen guten Überblick über den gegenwärtigen Stand der Forschung. Es kann allen, die an der modernen Entwicklung der Arterioskleroseforschung einerseits und den neuen Ergebnissen der Thrombozytenforschung andererseits interessiert sind, empfohlen werden.

U. BUCHER, Bern

G. MÖLLER (ed.) *Transplantation Reviews*, vol. 3, 4 and 5. Munksgaard, Copenhagen 1970. Dkr 40.-

Since the publication of the first volume of *Transplantation Reviews* in 1969, this series has rightly encountered a huge success. Its stated intention is to publish comprehensive and analytical reviews within the fields of clinical and experimental transplantation and cellular immunology. Under the editorial tutorship of G. MÖLLER, this intention has been well accomplished. Already now, many of the published papers are widely quoted. Of the 5 volumes published, the last 3 will be reviewed shortly.

Volume 3 (1970) deals with 'Strong and weak histocompatibility antigens', based on the proceedings of a one-day symposium in October 1969. Antigenic strength is a fascinating subject with theoretical and practical interest, the basis for 'strength' in transplantation still remains obscure as illustrated by the variety of hypotheses advanced to explain it. W. H. HILDEMAN and M. SIMONSEN presented introductory papers. These are followed by a stimulating and well edited general discussion. The topic, beside the fascinating theoretical aspects, presents considerable clinical interest, as still relatively little is known about the 'strength' of human transplantation antigens. This side of the topic is taken up in volume 4 (1970) 'Human transplantation antigens' by F. KISSMEYER-NIELSEN and E. THORSBY. This volume 4 gives a quite complete and competent review of the subject, including in the appendix a detailed account of the current methods in histocompatibility testing and evaluation of the results for human organ transplantation. These 3 volumes are in direct relation to transplantation. The next volume (vol. 5, 1970), brings papers and reviews more in relation to cellular immunology. They deal with lymphocytes and their capacity to bind antigen ('Antigen binding lymphocyte receptors'). The recognition of immunoglobulin like molecules on the surface of lymphocytes and their role in the reaction with antigen is most competently reviewed in this volume. All of the 6 papers present original data as well.

Even in a time when new journals and series creep up every year and one feels annoyed to have to look at more titles each time, this series fills a gap in treating competently topics which need to be reviewed and put in their proper context.

T. L. VISCHER, Basel

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